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EPIDEMIOLOGICAL STUDIES OF FRUCTOSAMINE IN RELATION TO DIABETES, CARDIOVASCULAR DISEASE AND MORTALITY

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Epidemiological studies of fructosamine in relation to diabetes, cardiovascular disease and mortality

THESIS FOR DOCTORAL DEGREE (Ph.D.)

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ABSTRACT

BACKGROUND: Diabetes is associated with an increased risk of micro- and macrovascular disease and mortality. Two main methods for diagnosis and control of type 2 diabetes are the measurements of blood glucose and glycosylated hemoglobin (HbA1c). These methods have some limitations where other techniques may complement. Hence, there is a need for other methods to be scientifically investigated and documented. Fructosamine is a biochemical marker of the amount of glycated proteins in the extracellular compartment of the blood and can serve as a complement to established blood markers of glycemic exposure. This thesis aims to evaluate fructosamine in relation to serum glucose and HbA1c and as a risk factor for type 2 diabetes (T2D), coronary heart disease (CHD) and death.

METHODS AND RESULTS: For all studies in this thesis, subpopulations of the AMORIS cohort (n=812,073) were used. **Study I.** In 871 subjects with a documented diagnosis of type 2 diabetes (T2D), the mean fasting fructosamine was 2.7 mmol/L and the mean value for fasting glucose and HbA1c respectively was 9.6 mmol/L and 7.2%. The linear correlation of fructosamine and HbA1c was high ($r=0.75$). Across three repeated measurements within one year, fructosamine and HbA1c followed the changes of fasting glucose over time. At a fructosamine level of 2.5 mmol/L, the sensitivity for the diagnostic criteria for diabetes was 61% and the specificity 97%. **Study II.** In 338,443 subjects, we observed an increased incidence of myocardial infarction or death from coronary heart disease (CHD) in subjects with higher levels of fructosamine. A fructosamine level of ≥ 2.7 mmol/L was associated with an increased hazard compared to a reference group of normoglycemic individuals (Adjusted HR=2.1 (2.0-2.2)). Comparable risk increases of CHD events and all-cause mortality were seen in groups of hyperglycemia defined by HbA1c. **Study III.** We investigated the risk of all-cause mortality based on fasting fructosamine levels over a study period of 27 years and included 215,011 subjects without diabetes at baseline. We observed a U-shaped mortality in relation to levels of fructosamine. The lowest decile of fructosamine was associated with an increased mortality (HR=1.20; 95% CI: 1.16-1.24) vs. a reference group (decile 2 to 9). The HR was decreased when we adjusted for haptoglobin. Analyses of cause-specific mortality showed increased risk ranging over several causes of death including an adjusted 42% increased mortality in lung cancer/COPD at low fructosamine levels. **Study IV.** We followed 296,436 subjects for diagnoses of T2D over a total study period of 27 years. We described trajectories of several metabolic risk factors. More than 20 years before a diagnosis of T2D, BMI and fasting glucose as well as triglycerides were increased compared to a matched control population. The average 20-year risk of T2D was 8.1% in this population. This risk was considerably increased with higher values of these factors.

CONCLUSIONS: The results from this thesis suggest that fructosamine levels are strongly associated with serum glucose and HbA1c and may be used as a complementary marker of glucose metabolism. High levels of fructosamine are associated with an increased incidence of myocardial infarction and death after adjustment for major cardiovascular risk factors. Several metabolic factors including BMI, fasting glucose, triglycerides and inflammatory blood markers, were increased in cases compared to matched controls more than two decades before a diagnosis of T2D. This suggests an early progression of the pathophysiology of T2D.

LIST OF SCIENTIFIC PAPERS

- I. **Malmström H**, Walldius G, Grill V, Jungner I, Gudbjörnsdottir S, Hammar N. Fructosamine is a useful indicator of hyperglycaemia and glucose control in clinical and epidemiological studies - cross-sectional and longitudinal experience from the AMORIS cohort. *PLOS ONE*. 2014;9(10):e111463.
- II. **Malmström H**, Walldius G, Grill V, Jungner I, Hammar N. Fructosamine is a risk factor for myocardial infarction and all-cause mortality - Longitudinal experience from the AMORIS cohort. *Nutrition, Metabolism and Cardiovascular Diseases* (2015), Vol. 25, Issue 10, p943–950.
- III. **Malmström H**, Wändell PE, Holzmänn MJ, Ärnlov J, Jungner I, Hammar N, Walldius G, Carlsson AC. Low fructosamine and mortality – a long term follow-up of 215,011 non-diabetic subjects in the Swedish AMORIS study. *Nutrition, Metabolism and Cardiovascular Diseases* (2016), Vol. 26, Issue 12, p1120–1128.
- IV. **Malmström H**, Walldius G, Carlsson S, Grill V, Jungner I, Gudbjörnsdottir S, Kosiborod M, Hammar N. Elevations of metabolic risk factors 20 years or more before diagnosis of type 2 diabetes – experience from the AMORIS study. *Manuscript submitted*.

LIST OF ABBREVIATIONS

ADA	American Diabetes Association
AMORIS	Apolipoprotein-related MOrtality RiSk
apoB	Apolipoprotein B-100
apoA	Apolipoprotein A-I
ARIC	The Atherosclerosis Risk in Communities (ARIC) Study
BMI	Body Mass Index
CHD	Coronary Heart Disease
CI	Confidence interval
COPD	Chronic Obstructive Pulmonary Disease
CVD	Cardiovascular Disease
DM	Diabetes Mellitus
HbA1c	Hemoglobin A1c
HDL-C	High density lipoproteins - Cholesterol
HR	Hazard Ratio
LDL-C	Low density lipoproteins - Cholesterol
MetS	Metabolic syndrome
MFR	National Medical Birth Registry
NPR	National Patient Register
NPDR	National Prescribed Drug Register
NDR	National Diabetes Registry
RCT	Randomized Clinical Trial
T1D	Type 1 diabetes
T2D	Type 2 diabetes

INTRODUCTION

From the time when Aretaeus of Cappadocia in the 1st century introduced and originally described diabetes in medical literature,¹ its methods of detection have been refined from only observable phenotypes via tasting of sweet urine to today's blood tests of the current circulating glucose as well as long-term measurements of glycated proteins.²

Still, only a few blood tests, which refer to the glyceimic pathways, are commonly available to clinicians and epidemiological researchers, mainly glucose measurements and glycated hemoglobin (HbA1c). Limitations of these methods might make them unreliable in many individuals and in several situations.³ Therefore, it could be of value to find complementary blood markers and methods for the purpose of risk prediction as well as for the control of diabetes including risk prognosis of coronary heart disease (CHD) and death.⁴

Fructosamine is a biochemical blood marker that is reflective of intermediate-term glyceimic exposure over about three weeks. It has been suggested that fructosamine may be a useful marker in the clinical diagnosis and control of diabetes.^{5,6} In addition, fructosamine might functioning well as a risk marker in epidemiological research. Yet, reports describing the role of fructosamine in risk prediction of severe cardiovascular events and mortality as well as its role in the long-term risk of type 2 diabetes are rare.

This thesis used data from the large AMORIS cohort with linkages to national health and mortality registers, and with an ample quantity of biochemical blood measurements including analyses of fructosamine. All measurements were performed in a standardized and well-documented way in nearly 500,000 individuals predominantly from the general working population in Stockholm, Sweden during 1985-1996. Hence, this cohort established a unique possibility to characterize fructosamine and its relations to established glyceimic blood markers, diabetes, cardiovascular disease and mortality.

The overall aim of this thesis is to increase current knowledge about fructosamine in relation to glucose and HbA1c, as a risk indicator for type 2 diabetes (T2D), coronary heart disease (CHD) and death respectively. The thesis also illustrates how long term metabolic status may alter from normal glyceimic conditions into diagnosed type 2 diabetes.

BACKGROUND

EPIDEMIOLOGICAL STUDIES

In epidemiological studies, the researcher investigates the distribution of diseases in human populations often with the objective to find causal factors. Epidemiological studies can be either experimental or observational. With a biochemical exposure, which is the focus of the present thesis, the experimental design with random assignment to certain levels is inherently impossible. Still, the main objective of an experimental design, e.g. a randomized clinical trial (RCT), and an observational design respectively, is comparable, i.e. to evaluate an effect of a given exposure of risk factors or a treatment for a specified disease outcome. In contrast to RCTs, in which risk factors assume a random distribution in the treatment group and the non-treatment group respectively, an observational design suffers from issues that origin in the non-random allocation to groups. The essential principles of an RCT, which are; to enable effect comparison (i.e. usage of placebo), to enable population comparison (i.e. randomization procedure) and to enable information comparison (i.e. blinding procedures), are however strived for and pursued by the epidemiologist in the definition process of relevant observed exposure contrasts.⁷ Through adherence to and recognition of those principles in the designing of the observational study, the validity of the observed associations increases.

DIABETES MELLITUS AND CARDIOVASCULAR DISEASE

Diabetes mellitus (DM) is a chronic disease, characterized and diagnosed by hyperglycemia. The disease frequently gives rise to various complications, some of them serious. Globally, the prevalence of DM is high, about 415 million individuals, and it is projected by the International Diabetes Federation (IDF) that 642 million people will have the disease in 2040.⁸

Cardiovascular diseases (CVD) refer to the circulatory system including the blood vessels and the heart. Coronary heart disease (CHD) is a major condition included in the general concept of CVD.⁹ The risk of CHD commonly increases in hyperglycemic and dyslipidemic conditions,¹⁰ which induce atherosclerotic disease progression. Hyperglycemia *per se*, may not cause atherosclerosis but is associated with several mechanisms, which are atherogenic.¹¹ Acute myocardial infarction is the ultimate stage of ischemic (i.e. oxygen restricted) conditions in the coronary arteries and in the myocardial tissue. Importantly, CHD is the leading cause of death globally.¹²

Cardiovascular diseases are responsible for a major proportion of the total mortality worldwide.¹² People with DM more often develop cardiovascular events compared to individuals with normal glucose tolerance.¹³ In addition, DM and cardiovascular disease

accounts for a large proportion of the health related burden of societies.¹⁴ Interventions to reduce incidence include life style changes such as increased physical activity, healthier dietary habits, smoking cessation and pharmacological treatment of various conventional risk factors like hyperglycemia, blood pressure and dyslipidemia.

Environmental exposures as those above, in combination with genetic factors¹⁵ may affect levels of biochemical blood particles, and consequently the risk of disease and complications.

Types of diabetes

The majority of individuals with diabetes can be categorized into two types with different etiology.¹⁶ One is with autoimmune etiology (type 1 diabetes (T1D)) and the other non-insulin dependent and non-autoimmune (type 2 diabetes (T2D)). In more recent decades, it has been realized that diabetes classification into autoimmune T1D and non-autoimmune T2D cannot be based (as previously) primarily on phenotypes,¹⁷ such as insulin-dependent and non-insulin dependent diabetes but rather on the presence or absence of immunological markers for autoimmune disease. By biochemical criteria, non-insulin dependent individuals with diabetes who possess diabetes-specific antibodies (such individuals are commonly termed as LADA) would be classified as type 1 diabetes. In addition, the monogenic forms of MODY (Maturity Onset DM in Young) now single out from T2D. Genetic factors play an important role for T2D and for T1D.¹⁷ For all

forms of diabetes, the risk of disease development is a combination of environmental and genetic factors.¹⁸

Proportionally, T2D roughly accounts for 90% of all individuals with diabetes.

BIOCHEMICAL RISK MARKERS FOR DIABETES, CVD AND MORTALITY

There are a number of glycemic, lipid related and inflammatory markers, which are important in casual pathways and/or in risk prediction of T2D and CVD. Brief descriptions of such markers included in this thesis are given below.

Glycemic exposure

Glucose

High levels of circulating blood glucose is inherently a factor of importance for diabetes because of its essential and defining role in the diagnosis criteria.^{13,19-22} Increased glucose levels act as an indicator either for non-functioning insulin production in the pancreatic beta cells and/or for reduced uptake of glucose in the body. Different interpretations of the glucose test are crucial, is it made in a fasting or a non-fasting state. In the fasting state, i.e. overnight fasted for at least eight hours, the glucose test reflects the hepatic production of glucose. Postprandial or after an externally glucose load, the test reflects the body uptake or the increase of glucose following a glucose load. Hence, the fasting glucose and glucose measured postprandial urge physiologically different interpretations.²³ Fasting plasma glucose as a diagnostic marker has some limitations;

fasting status must be assured, long-term control of glucose needs to be performed by frequent measurements and intra- as well as intervariability could be substantial.²⁴

HbA1c

HbA1c, formed via intracellular irreversible glycosylation of the hemoglobin, which is abundant in the red blood cells, has its usage as a long-term indicator of glucose exposure.^{2,24} It reflects the glycosylation over the whole lifetime of the red blood cell (120 days and 60 days for hemoglobin) with the most recent month emphasized.²⁵ Diseases affecting the red blood cells, e.g. hemolytic anemia, sickle cell anemia and impaired red blood cell turnover, can severely influence the accuracy of the HbA1c measurement.²⁵ The test is somewhat more technically difficult to analyze compared to standard plasma tests and therefore more expensive to perform than an ordinary plasma glucose test. Historically, one consideration for suggesting an important role of HbA1c in diagnostics and risk prediction of diabetes and its complications was its strong association with microvascular diseases, including retinopathy.²⁶⁻²⁸

Non-traditional biomarkers of glycemic exposure

Among non-traditional markers of glycemic exposure, fructosamine (more on page 23 ff.), glycated albumin and 1.5-Anhydroglucitol are debated.²⁹ In contrast to fructosamine, glycated albumin is a test targeted to measure only the proportion of serum albumin being

glycated. The 1.5-Anhydroglucitol measures the filtration of monosaccharides through the kidney and mirrors average glycemia in the preceding 2 to 14 days.³⁰ With regard to the test of 1.5-Anhydroglucitol, it is uncommon in clinical practice and is expensive compared to measurements of fructosamine or glycated albumin.²⁹

Lipids and lipoproteins

Serum/Plasma Cholesterol

High levels of total cholesterol increases the risk of coronary heart disease (CHD) and death.³¹ The cholesterol may express atherogenic as well as protective effects on the vasculature depending on the type of lipoprotein responsible for transport. Cholesterol transported in low-density lipoprotein (LDL-C) penetrates the arterial wall and may exhibit atherogenic effects. In contrast, when transported in high-density lipoproteins (HDL-C), the cholesterol serves anti-atherogenically, hence, HDL-C works protectively on the risk of cardiovascular outcomes.¹³

Serum/Plasma Triglycerides

The direct effect of triglycerides in the promotion of CVD has been argued for decades. Yet, descriptive associations of triglycerides and CVD have been shown in several studies.³² Through its close inverse association to HDL-C, higher triglycerides are frequently observed in individuals with CVD and T2D. Reasons for the controversy about direct effects of triglycerides on CVD may be rooted in methodological issues and

in modest effects from those studies that yet suggested an association.³² It has been shown that independent effects of triglycerides are marginalized when also controlling for HDL-C, however the effect of HDL-C remains.³³ The combination of high triglycerides and lower HDL-C is characteristic for atherogenic dyslipidemia³⁴ and for T2D. In addition, several other risk factors for both T2D and CHD are associated with high triglycerides^{35,36} and thus it is important to assess the overall atherogenicity of plasma in hypertriglyceridemia.³² Today, strong evidence suggests a casual role for triglycerides in CV disease.³⁷⁻³⁹

Apolipoproteins

Cholesterol and triglycerides are transported in blood embedded in lipoproteins, which are mainly classified as very low density (VLDL), intermediate density (IDL) low density (LDL) and high-density (HDL) lipoproteins respectively. Attached to each VLDL, IDL and LDL particle is one apolipoprotein B-100 (apoB). Thus, the sum of apoB particles, especially those attached to small dense LDL-particles, reflects the transport of potentially atherogenic cholesterol and triglycerides. Therefore, apoB has an atherogenic effect and has been demonstrated to be a strong risk factor for CVD outcomes^{40,41} and to have similar effect as to that from LDL-associated cholesterol.^{42,43} Further, in younger ages, apoB has been reported to strike harder.⁴³ On the other hand, apolipoprotein A-I (apoA) attaches to the anti-atherogenic HDL particle

and higher levels of apoA are more protective. There are suggestions that the ratio of apoB and apoA would serve as the best predictor of severe CHD because of its balancing of atherogenic and anti-atherogenic characteristics.⁴⁴⁻⁴⁶

Inflammatory related exposure

Albumin

Albumin is richly present in serum and has antioxidant properties. It is a negative acute-phase protein⁴⁷ and low serum albumin levels are reported to increase the risk of CVD.⁴⁸⁻⁵⁰ In addition, changes in serum albumin levels has been shown to have protective effects in the development of the metabolic syndrome (MetS).⁴⁷

Haptoglobin

Haptoglobin is an acute phase glycoprotein and is activated in protection to oxidative stress.⁵¹ Mainly it is synthesized from hepatic cells, but also exists in fat tissue. Elevated haptoglobin levels in serum are seen under acute inflammatory conditions, however chronic illness may also increase the haptoglobin levels and it has been linked to both diabetes and obesity^{51,52} as well as the MetS.⁵³ Higher levels of haptoglobin have been associated with higher CVD risk.⁵⁴⁻⁵⁶

Uric acid

Uric acid (or urate), accumulates while purine compounds are broken down. It is an end-product of this metabolism.⁵⁷ Most often, the capacity of the kidney glomerulus is the limiting factor in the appearance of high uric

acid levels. Uric acid has been associated with development of T2D and possesses inhibitory effects on the insulin sensitivity.⁵⁸ Its casual effect on CVD is disputed⁵⁷ because of its close association to other CVD risk factors.⁵⁹

RISK FACTORS AND DIAGNOSIS OF TYPE 2 DIABETES

Risk factors for T2D and CVD

The risk of T2D increases with higher age and overweight/obesity as well as dyslipidemia, factors which may be associated with increased fasting blood glucose.^{13,60} These factors commonly appear clustered and are components of the metabolic syndrome (MetS),⁶¹ which also includes hypertension.^{13,62} The risk of T2D increases five-fold with the presence of MetS and cardiovascular events are markedly more common with the syndrome.⁶² Thus, most people diagnosed with T2D have one or more of the factors included in the MetS. The prevalence of MetS worldwide is increasing in parallel with a more sedentary life style, increasing overweight and abdominal obesity.⁶² With regard to T2D, other risk aspects to consider include family history of DM, ethnicity, tobacco usage and polycystic ovary syndrome in women.⁶³ Dyslipidemia in subjects with T2D typically include hypertriglyceridemia, low HDL-C and normal LDL-C concentration.⁶⁴ Although low LDL-C may be found in subjects with T2D, those subjects many times have a higher amount of small dense LDL particles.^{64,65} Such particles more easily penetrate the arterial wall,

eventually leading to atherosclerotic plaque building. In relation to small dense LDL, those subjects often have high apoB (atherogenic) levels and low apoA-1 (atheroprotective) levels, leading to high apoB/apoA-1 ratio, a very strong risk factor for MI and stroke.^{40,44,45}

Risk prediction models for T2D

Several risk prediction models have been developed for the identification of individuals at increased risk of T2D.⁶⁶ These models are based on non-modifiable risk factors, such as age, sex, ethnic origin and family history of DM, but also on modifiable factors, including smoking, body mass index (BMI), dietary and alcohol habits, exercise, and lipid metabolism. Today, several models, using simple commonly available clinical characteristics, are used and show good prediction ability. The additional benefit of adding alternative novel blood markers to available risk prediction models is unclear.⁶⁶ In a recent review article, only one non-glycemic biomarker, uric acid, was considered to have high predictive value (in addition to age, sex, BMI, smoking, family history and hypertension) for future T2D, whereas many biomarkers were shown to have low or moderate association with risk of T2D.⁶⁷ Nevertheless, by using Mendelian randomization technique, uric acid was considered non-causally associated with T2D.⁶⁸ Similarly, triglycerides levels did not show evidence of a casual relation to T2D.⁶⁹

Diagnostic criteria of diabetes

The diagnosis and control of DM are based on blood biomarkers. Diagnostic criteria defined in guidelines from American Diabetes Association (ADA), European Association for the Study of Diabetes (EASD) and the World Health Organization (WHO) form basis for combinations of fasting glucose, HbA1c and 2-hour glucose measured after an oral glucose tolerance test (OGTT).^{13,19-22}

In common, for all these diagnostic criteria is a fasting plasma glucose of ≥ 7.0 mmol/L (measured once or twice according to different guidelines) for the diagnosis of DM. OGTT with a 2hPG of ≥ 11.1 mmol/L is another way to establish a diagnosis of diabetes but are mainly for feasibility reasons less often used.¹³ A third parameter with which to diagnose diabetes has been added by ADA and WHO, i.e. an HbA1c of 6.5% (48 mmol/mol). It should be noted that diagnostic criteria have been, and are still under debate and that diagnostic limits for fasting glucose have been lowered in recent years.⁷⁰⁻⁷²

Furthermore, levels of fasting glucose in subjects without diabetes have shown a positive association with increased risk of CVD and mortality.^{73,74}

Early identification of type 2 diabetes

For prevention purposes, it is of importance to early identify people of increased risk of developing T2D. The disease may gradually develop over several years preceding the diagnosis as reported in studies describing pre-diagnostic trajectories of metabolic risk

factors.⁷⁵⁻⁷⁷ Differences in fasting glucose between cases and controls have been observed up to 13 years before the diagnosis.⁷⁷ This progression over time has predominantly been shown for fasting and 2-h plasma glucose, HbA1c and triglycerides.⁶⁶ Early identification of prediabetes is desirable, since the development into overt disease is largely preventable or possible to delay.^{78,79}

Screening for T2D has been demonstrated to gain cost efficiency and number of quality adjusted life years (QALYs).^{80,81} Although one large European randomized clinical trial initially reported no benefit of screening followed by intensive treatment versus no screening on all-cause-, cardiovascular-, cancer- or- other causes of mortality, over 10 years,⁸² the authors later concluded that screening for T2D could be feasible but it is a challenging endeavor.⁸³ Furthermore, follow-up studies have shown, by simulation techniques, a reduced rate of cardiovascular events and all-cause mortality following screening with intensive treatment of detected cases.⁸⁴

Risk scores for predicting T2D may be useful in clinical practice. The number of scores published has increased dramatically from the year of 2000, and many are mainly based on social and behavioral characteristics rather than on biomarkers.⁸⁵ Furthermore, only a small proportion, whereof the Finnish Diabetes Risk Score (FINRISC) is widely recognized,⁸⁶ of all developed risk scores for T2D are used in clinical practice, likely due to poor validation and calibration.⁸⁵

FRUCTOSAMINE

Historical background

Fructosamine, a ketoamine, was first synthesized in 1886 and was given the chemical reference “1-amino-1-deoxy-fructose”.^{87,88} Although it was synthesized, the definition of fructosamine as a general term for glycated serum proteins, was not until 1982 introduced into the chemical literature.⁸⁹ A few methods for measuring fructosamine were available, including affinity chromatography, thiobarbituric acid colorimetric procedure (TBA) and Nitroblue Tetrazolium Colorimetric procedure (NBT). Early, all of these methods demonstrated good discrimination of people with insulin dependent diabetes from individuals with normal glucose levels.^{87,90}

Biochemical aspect

Fructosamines are particles formed in non-enzymatic processes, primarily because of glucose bindings to circulating serum proteins.^{29,87} Fructose is proportionally low in the circulation because it is removed by hepatic processes,⁹¹ and therefore only a minor part of fructosamine origins from fructose bindings. Yet, a more rapid reaction with protein has been reported for fructose compared to glucose.⁹² Albumin constitutes a large proportion (up to 80%) of circulating proteins. Hence, a measurement of only glycated albumin does not account for all proteins being glycated. Immunoglobulins, lipoproteins and apolipoproteins as well undergo glycation and therefore are proportional quantities of fructosamine

particles.^{93,94} The half-life ($T_{1/2}$) of the most ample protein in blood, albumin, is shorter than the corresponding $T_{1/2}$ for hemoglobin, thus, fructosamine reflects a shorter period of glycemic exposure compared to HbA1c. Fructosamine can be considered to be an intermediate-term measurement of glycemic exposure across the preceding 1-3 weeks⁸⁷ compared to HbA1c, which has a considerably longer accumulation rate of 8-12 weeks.

Clinical aspect

Glycation of proteins that occurs in the extracellular matrix, i.e. the forming of fructosamine (extracellular glycation of proteins), may reflect different pathophysiological processes compared to intracellular glycation measured by HbA1c. Fructosamine converges to mean blood glucose over a shorter time compared to HbA1c. In certain situations, the shorter period a fructosamine test reflects may be an important complementary biomarker. This could be valid in gestational diabetes (GDM),^{94,95} in the evaluation of treatment intensification/de-escalation and in situations where earlier risk indications of various deleterious conditions warrant attention. In addition, evaluation of treatment response in clinical pharmacological studies may value a more rapid assessment of change. Some clinical limitations of the fructosamine test have been reported (e.g. in conditions of hypo- and hyperthyroidism, in myeloma and in nephrotic syndrome),⁹⁶⁻⁹⁹ which might limit its use. During pregnancy, dilutional

anemia may develop that might affect fructosamine negatively to lower levels not accurately reflecting current glycemia. Therefore, authors have reported that fructosamine may be unfavorably to use in investigation of GDM. Glycated albumin, which is not affected by diluted serum would be better recommended as a preferred test.⁹⁴ Despite a few limitations, fructosamine is a simple test measured in serum, fasting is not needed, it is inexpensive, and it is insensitive to any disorders of the red blood cells.²⁹ Altogether, fructosamine could be useful as a complementary biomarker in the clinical setting but more research guiding its applicability in different situations is warranted.

Research observations in previous studies on fructosamine

Descriptive associations

Early research showed strong correlations between fructosamine, HbA1c and glucose.¹⁰⁰⁻¹⁰⁴ Some reported that non-linear correlations would be more appropriate to describe the associations.¹⁰³ Most of these studies were of limited size and performed in subsets of subjects with different stages of diabetes and other medical conditions. More recently, the ARIC study reported correlations of the same magnitude as previously seen, between fructosamine and HbA1c.¹⁰⁵

Efforts to show associations of fructosamine with other factors (e.g. age, ethnicity, and sex, family history of T2D, BMI, hypertension, cholesterol and smoking) described an inverse association with BMI in patients with

diabetes.^{25,106-110} In normoglycemic subjects, high values of fructosamine were more associated with being a man and with higher age.¹⁰⁶ Furthermore, high fructosamine levels in subjects without diabetes were more common in individuals of African-American origin.²⁹

Cardiovascular outcomes

Fructosamine has not until recently been linked to risk of CVD. In 2015, Selvin and colleagues found an increased risk of CVD in individuals with elevated fructosamine.¹⁰⁵ The study, based on subjects from the ARIC cohort, included both subjects with and without diabetes. Among subjects with no diagnosed DM, the study showed a 33% increased adjusted hazard for CHD, in the top percentiles of fructosamine (≥ 2.64 mmol/L), compared to a reference group. Restricting to subjects with diagnosed DM, a fructosamine of ≥ 2.70 mmol/L, which roughly corresponded to an HbA1c of 7%, was associated with an increased hazard of CVD outcomes and death compared to the same previous used reference group. Furthermore, diabetes patients below 2.70 mmol/L also showed a significant increased hazard compared to the reference group and the hazard ratio was of greater magnitude than that reported for subjects without diabetes who had higher fructosamine. In this study, by using identical percentile thresholds, fructosamine showed higher hazard ratios for CVD as compared to HbA1c.¹⁰⁵

The study by Selvin et al. showed an association between fructosamine and CVD

in a population based study and discussed fructosamine levels of 2.60-2.70 mmol/L as relevant thresholds for increased risk.¹⁰⁵ In contrast to this study, the authors of a Finnish study with only diabetes-free individuals found no significant difference in CVD risk between the highest and lowest quartile of fructosamine.¹¹¹

Mortality

In the ARIC study, high fructosamine levels were associated with increased all-cause mortality.¹⁰⁵ This association remained after multivariate adjustment for traditional CVD risk factors and was particularly strong in T2D patients. The Finnish study observed no association between fructosamine and all-cause mortality.¹¹¹

It has been reported that low fasting glucose levels may be associated with an increased mortality among individuals without DM.¹¹² In addition, this association was shown for low HbA1c levels and all-cause or CVD mortality.^{113,114} Whether low levels of fructosamine relate to a higher mortality has not been studied in detail but recent research suggests an elevated risk in the lowest range of fructosamine.¹⁰⁵

Progression of T2D

Glycation of proteins occurs continuously in the circulatory system. The concentration of fructosamine typically increases with elevated plasma glucose concentrations and generally remains high in manifest DM.⁸⁷ A limited number of studies have investigated the association between glycated serum proteins

and DM^{103,115-117} and reported racial physiological differences in sensitivity of glycation.¹¹⁸⁻¹²¹

One recent paper from the ARIC study found a five-fold increased risk of DM for individuals in the top percentiles of fructosamine, (≥ 2.64 mmol/L).¹¹⁵ This cohort study observed almost one thousand new cases of DM during the follow-up. A cohort study with fewer new cases of DM during a short follow-up, observed a four-fold increased risk for people in the top quartile of fructosamine (≥ 2.41 mmol/L), compared to people in the lowest quartile. The risk was much weaker after adjustment for HbA1c or glucose.¹¹⁷

In addition to these two cohort studies, cross-sectional studies have described higher fructosamine levels in people with known DM compared to people with no DM.^{103,116,118} One study did not show any excess risk of higher fructosamine and furthermore reported an U-shaped risk curve.¹²² The authors suggested that genetic variation in glycosylation pathways was a possible explanation for this null association.^{122,123}

CURRENT KNOWLEDGE GAP

Fructosamine has not previously been extensively investigated with regard to T2D, micro-and-macrovascular complications and mortality. Strong correlations to the well-known biochemical measurements of diabetes, i.e. fasting glucose and HbA1c, have been demonstrated. However, these were studies of limited size and in selected populations. Reports are inconclusive

regarding the association of fructosamine and the incidence of CVD and mortality. Rarely the complete range of fructosamine is described and careful evaluation of risks at high as well as low levels is needed. The usefulness of fructosamine as a long-term predictor of T2D needs further investigation in a large population with long follow-up.

AIMS OF THE THESIS

OVERALL AIM

The overall aim of this thesis is to investigate fructosamine in relation to other major biomarkers of glucose metabolism, type 2 diabetes, cardiovascular disease and mortality.

Specific aims

- a) To evaluate cross-sectional and longitudinal relationship between fructosamine and the established indicators of hyperglycemia; serum glucose and HbA1c.
- b) To evaluate the association of increased fructosamine levels with the incidence of acute myocardial infarction and all-cause mortality.
- c) To evaluate the association of low fructosamine levels and mortality in a population without diagnosed diabetes.
- d) To evaluate the long-term prediagnostic development in metabolic risk indicators, including fructosamine, before a diagnosis of type 2 diabetes.

MATERIALS & METHODS

THE AMORIS COHORT

All papers in this thesis were based on subjects from the AMORIS (Apolipoprotein-related MOrtality RISk) cohort that has been described extensively elsewhere.^{44,124} This cohort included 812,073 subjects (49% men and 51% women) with a mean age of 42.6 years at the first health examination during 1985-1996. The majority of the cohort subjects was living in Stockholm County, Sweden, at the time of inclusion and the sex, socioeconomic and ethnic distribution of the cohort is representative to that of the general Stockholm population in 1990.¹²⁴

Reasons for referral

All subjects had a health examination with blood sampling because either they were out-patients or they attended a routine part of

health check-ups through occupational health care. Pay codes indicated the referral reason for the blood sampling. In this thesis, a core population, those with available glucose measurement, was utilized (n=551,768). In this population, three pay codes were used for the majority (77%) of the subjects; health check-up paid by the employer (34%), health check-up paid by the Swedish Social Insurance Agency (SSIA) (22%) and referral by general practitioners (21%). The reasons for the remaining visits (24%) were for the purpose of validation or were unknown. Subject characteristics of demographic, socioeconomic and biomedical factors were similar for all three types of pay codes (Table 1).

Table 1. Baseline Characteristics of subjects stratified by referral code

	Health check-up (paid by employer)	Health check-up (paid by SSIA ^a)	Referral by physician	Unknown referral reason
N	185,378	121,370	113,652	123,562
Age (years)	42	43	48	46
Fasting	48%	42%	48%	38%
Female	40%	45%	56%	44%
Low socioeconomic status	44%	48%	37%	44%
High socioeconomic status	49%	46%	37%	49%
BMI (kg/m²)	24.4	24.8	24.1	24.4
Serum glucose (mmol/L)	4.9	4.9	5.1	5.0
Fructosamine (mmol/L)	2.07	2.08	2.08	2.28
Total cholesterol (mmol/L)	5.4	5.5	5.5	5.7
Triglycerides (mmol/L)	1.3	1.4	1.3	1.3

^aSwedish Social Insurance Agency

Laboratory analyses

More than 35 million laboratory values, including repeated measurements, were recorded in subjects of the AMORIS cohort, covering more than 500 biomarkers. All measurements were done on fresh blood at the same laboratory (CALAB, Stockholm, Sweden) with a well-documented methodology. Several well-established chemistry biomarkers among others triglycerides, total cholesterol, creatinine and serum glucose were part of a standard analysis package offered without additional cost. In addition, novel analyses were included although not requested by the referring physician, e.g. markers for specification of atherogenic dyslipidemias (apoB, apoA and the apoB/apoA-I ratio) and fructosamine for indication of glycemic

exposure. These analyses were not clinically recognized as recommended analyses, but the CALAB laboratory performed investigations on potentially valuable risk markers for future use, as they were an international leader and frontrunner for the development of health screening and automation in laboratory practice (Autochemist®). Hence, the fructosamine assay, determined with the Nitroblue Tetrazolium Colorimetric procedure (NBT), was measured in 456,383 individuals, with a coverage across many different levels of glycemic exposure. Among those who had measured fructosamine, 43% had at least two repeated measurements at different dates and many individuals had three or more visits (Table 2).

Table 2. Biochemistry serum markers commonly measured in the AMORIS cohort and included in the present thesis

Serum marker	N		% subjects with	
	≥1 visit	≥2 visits	≥3 visits	≥4 visits
Creatinine	575,196	46	25	15
Cholesterol	574,251	46	25	15
Triglycerides	572,162	46	25	15
Glucose	551,768	45	25	15
Albumin	526,417	45	24	15
Uric acid	531,347	45	24	15
Fructosamine	456,383	43	22	13
Haptoglobin	444,314	42	21	12
apoB	183,609	31	14	8
apoA	196,890	30	14	8
HbA1c (blood)	24,863	47	31	22

Associations of fructosamine in the AMORIS population

In almost one million analyses of fructosamine performed in 456,383 individuals in the AMORIS cohort, the levels were virtually normal distributed (Gaussian distribution) with a mean value and standard deviation of 2.09 and 0.29 mmol/L respectively. Values below 1.8 mmol/L and values above 2.6 mmol/L were uncommon (Figure 1). There were only few subjects who had values above 4.0 mmol/L or values below 1.0 mmol/L (<0.4%). On average, men had somewhat higher levels compared to women. No difference was seen, were the subjects fasting or non-fasting (Figure 2). A weak positive linear correlation was noted between fructosamine and age ($r=0.15$) and hence slightly increased fructosamine was seen by increasing age in a sex and fasting adjusted linear regression model. Fructosamine

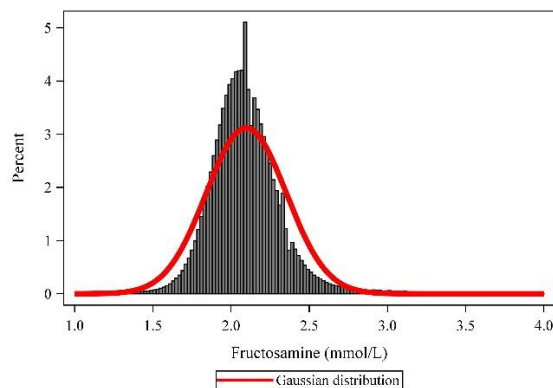


Figure 1. Distribution of fructosamine in the AMORIS population, compared to an estimated Gaussian distribution.

increased significantly by 0.0033 mmol/L for each year the age increased.

Metabolic blood markers of glycemic, lipid related and inflammatory exposure also demonstrated significant linear correlations with fructosamine and were observed for glucose ($r=0.53$), total cholesterol ($r=0.25$), triglycerides ($r=0.25$) and albumin ($r=0.17$) (Figure 3). Fructosamine was mainly associated with the atherogenic part of cholesterol as shown by a positive association with apoB and no association with apoA. Non-linearity was noted for fructosamine and most markers at very low or high levels. Nonetheless, in reasonable intervals of each marker (99.8 % of all subjects) a positive linearity prevailed. Serum albumin, which has burdened the potential use of fructosamine,^{3,125-127} was rather uncorrelated with fructosamine in the hyperglycemic interval.

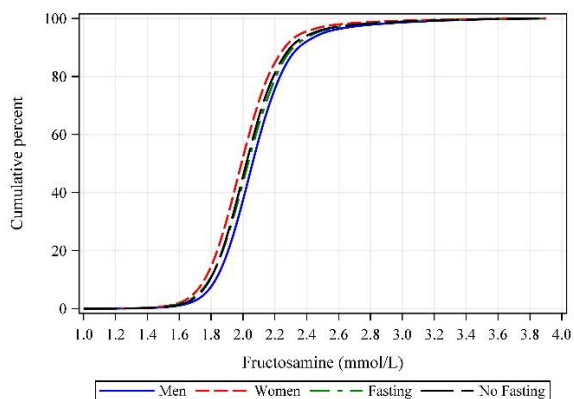


Figure 2. Cumulative distribution of fructosamine in the AMORIS population. Shown for men, women, fasting subjects and non-fasting subjects respectively

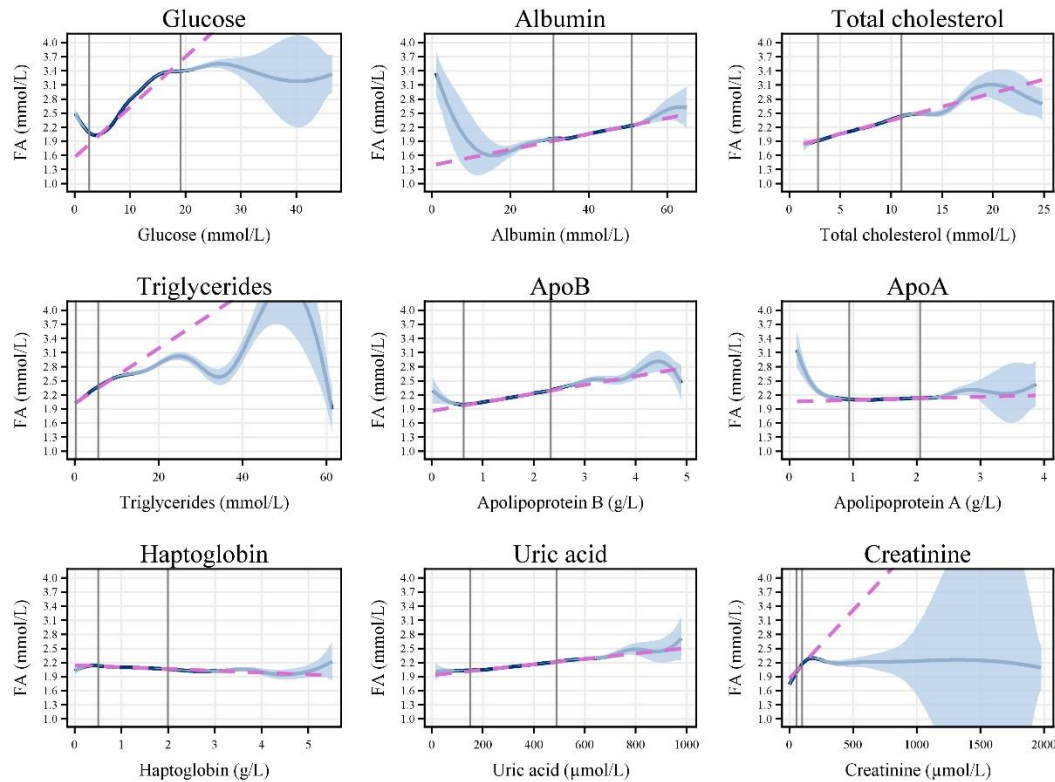


Figure 3. Relationship between fructosamine (FA) and biochemical serum markers in fasting subjects. Dashed line: linear regression in fructosamine range 1.0-4.0 mmol/L. Solid blue line: penalized B-spline with 95% CI for the mean. Vertical lines represents thresholds for 0.1%- extremes of the population.

REGISTER DATA LINKED TO AMORIS

Loss to follow-up can be an important limitation in epidemiological studies. In Sweden, there are national health and population registers of high quality and completeness for hospitalizations, specialized outpatient care, dispensed prescriptions of medical drugs, migration and mortality. The unique Swedish personal identification number (PID) enables linkage of research studies to these national records. This helps to minimize loss to follow-up in epidemiological studies using these registers.

Within the scope of the ethically approved (*Record number: 2010/1047-31/1*) research project “*Epidemiologic studies of metabolic*

factors and inflammation in relation to chronic disease”, the CALAB laboratory database was linked to 24 registers. These included national health registers, quality of care registers, national registers of socioeconomic data and research cohorts (Table 3). During the period, 1985-2012, there were 153,820 deaths (18.9%), 175,334 cancer diagnoses (21.6%) and 4.5 million hospitalizations recorded for the cohort.¹²⁴ Furthermore, about 48,000 new cases of T2D occurred in the sub population with serum glucose measured during this period.

The registers linked to the AMORIS cohort that are essential for this thesis are briefly described below.

Table 3. Important register linkages to the AMORIS cohort

Category	Data Source	Linkage period
CALAB blood sampling	AMORIS	1985-1996
National Health Register	Inpatient Care	1964-2011
	Specialized Outpatient Care	2001-2011
	Cause of death	1985-2011
	Prescribed drug ¹²⁸	2005-2012
	Medical Births (MFR)	1973-2011
Migration, Social, Family	Migration	1968-2012
	Census/LISA	1970-2010
Lifestyle	National surveys and research cohorts at Karolinska institutet	1963-2012
Quality of Care	National Diabetes Registry (NDR)	1996-2012
	SWEDHEART	1991-2012

National patient register

The Swedish National Patient Register started regionally in 1964 and has national coverage since 1987.¹²⁹ Stockholm County was one of the first regions with a patient register starting in 1970. Initially, only inpatient care visits were recorded, however from 2001 the register also records all specialized outpatient care visits. The Swedish National Board of Health and Welfare performs regular updates of this register, which has coverage of more than 99% of all somatic and psychiatric hospital discharges including patient data, geographical data, administrative data of the hospital stay, and medical data. The diagnoses are defined by codes standardized in the *International Classification of Diseases* (ICD).

National cause of death register

The Swedish Cause of Death Register started in 1961 and comprise both specific causes of death and date of death for all Swedish

citizens at the time of death, regardless if death occurred in Sweden or elsewhere. The Cause of Death Register use coding in accordance with the ICD.

National diabetes registry

The Swedish National Diabetes Registry (NDR) started to include patients with DM in 1996. In the beginning, the case coverage was limited but today it roughly includes almost 90 % of all Swedish diabetes patients. The estimated coverage differs across Swedish County Councils and some counties estimate having full coverage.¹³⁰ The NDR is an important source for DM research in Sweden and includes information about type of DM, year of diagnosis, current glycemic and lipid levels and examinations and assessments of the retina. Through this register, it is possible to identify prevalent as well as incident DM patients.

Lifestyle

Lifestyle and comorbidity factors such as smoking status, anthropometric measurements, self-reported diseases, including DM and hypertension, were obtained through linkages with research cohorts, setup and maintained by research groups at Karolinska institutet. This includes the WOLF study,¹³¹ the Stockholm 60-years old cohort,¹³² the Swedish Twin Registry,¹³³ Sollentuna Prevention Program,^{134,135} and the COSM/SMC nutritional cohorts.¹³⁶ In the scope of this thesis, the author integrated information on important covariates from several of those registers (e.g. smoking, hypertension and BMI) in a standardized covariate database. This database enabled and simplified the inclusion of potential confounders in the analyses.

Migration and social factors

The Migration Register maintained and updated by *Statistics Sweden* (Statistiska centralbyrån, SCB) provided dates for immigration and emigration. Those dates enabled censoring at emigration out of Sweden and consequently lost to follow-up. Socioeconomic status was derived from occupational codes recorded in the national censuses from 1970 to 1990 and was available for nearly all subjects in the AMORIS cohort. Information on occupation and education was also available from the “*Longitudinal integration database for health insurance and labour market studies*” (LISA) in 1990 and later.

STUDY METHODS

Short descriptions of the methods, which were used in the four papers are given below. These are also summarized in Appendix Table 1.

Study I

Participants

The study population was identified from the AMORIS cohort and comprised those subjects who had glucose, fructosamine and HbA1c measured at the same examination date during the period 1985-1996 (n=10,987). In the study population, 53% were women.

Exposure

We identified subgroups of glycemic exposure based on ADA guidelines or on documented diabetes diagnosis primarily supplied by the National Diabetes Register (NDR). We defined five groups; normal glucose tolerance, prediabetes, newly diagnosed T2D (NewT2D, through the AMORIS blood sample), previously diagnosed T2D (DiagT2D, documented in register) and T1D (from register or less than 30 years and diabetes diagnostic levels at the AMORIS blood sample).

Statistical analysis

We estimated Pearson linear correlation coefficients for combinations of fructosamine, glucose and HbA1c and analyzed correlations in fasting as well as non-fasting conditions. Furthermore, we presented correlation coefficients overall and within the glycemic exposure subgroups. Partial correlations were estimated to account

for potential confounder influence on the estimated correlations. Sensitivity and specificity for diabetes at defined cut-off values for fructosamine and area under the receiving operator curve (AUC-ROC) were estimated by use of a “gold standard” for diabetes diagnosis based on fasting glucose and HbA1c (ADA). In a subset with at least three repeated measurements on all three markers available within one year, a longitudinal analysis was conducted to evaluate the associations progressively.

Study II

Participants

In the AMORIS cohort, all subjects who were 30 years or older and who had fructosamine, glucose, total cholesterol, triglycerides and serum albumin measured simultaneously during the baseline period 1985-1996 and had no previous history of CVD were included in the study population (n=338,443; 178,947 men and 159,496 women).

Exposure and other potential confounders

We categorized fructosamine levels in accordance with clinically relevant subgroups based on glycemic exposure levels observed for glucose, HbA1c and fructosamine in study I (Table 4).¹³⁷

Table 4. Classifications of fructosamine levels.

Fructosamine (mmol/L)	Classified as:
< 1.78	Lowest 5%
1.78-2.29	Normal glucose (ref)
2.30-2.59	Prediabetes
2.60-2.69	Well-controlled T2D
≥ 2.70	Poorly controlled T2D

Outcome and follow-up

The primary outcome in this study was incident myocardial infarction (MI). Non-fatal MI and death from coronary heart disease (CHD) were included in the primary outcome. Secondary outcome was all-cause mortality. We obtained outcomes from linkage to the national inpatient register and cause of death register respectively. Subjects were followed from the first examination (1985-1996) until first MI, death, emigration or the end of the study (December 31st, 2011), whichever occurred first.

Statistical analysis

We used Cox proportional hazard regression,¹³⁸ with attained diurnal age as the underlying timescale, to estimate hazard ratios with 95% confidence intervals for MI and all-cause mortality respectively comparing exposure categories of fructosamine to the reference group. The analysis allowed for updating exposure and other covariates whenever repeated measurements became available (i.e. repeated measurements). To disentangle the independent effect of fructosamine, we tested four models. Model 1: sex, age and calendar time; Model 2: Model 1+ total cholesterol, triglycerides and serum albumin; Model 3: Model 2+social class; Model 4: Model 2+ glucose. In addition, we compared three measures of glycemic exposure (i.e. fasting glucose, HbA1c and fructosamine) with respect to risk prediction of the study outcomes.

Study III

Participants

All subjects who were fasting and had simultaneous measurements of fructosamine, glucose, total cholesterol, triglycerides, albumin, creatinine, uric acid and haptoglobin in the baseline period of 1985-1996 (n =215,011; 47% women). Patients with diabetes were excluded at baseline and censored whenever a diagnosis became known during the follow-up period.

Exposure and other potential confounders

The 10% lowest ordered fructosamine constituted the exposure of interest. Ordered levels between 10 and 90% established the reference category and the highest 10% constituted an 'other' category. Potential confounders included metabolic biomarkers (i.e. total cholesterol, triglycerides, albumin, creatinine, uric acid and haptoglobin). Sensitivity analyses included smoking (ever/never), BMI and reverse causation of malignancies (by linkage to the National Cancer Register).

Outcome and follow-up

We obtained information on all-cause mortality and cause-specific mortality respectively from the national cause of death register. We defined five categories of cause-specific deaths: 1) cardiovascular, 2) cancer, 3) lung cancer/COPD, 4) infections and 5) all other deaths. Subjects were followed from the baseline examination (1985-1996) until death, emigration or the end of the study (December 31st, 2011), whichever occurred first.

Statistical analysis

Cox regression models¹³⁸ were constructed by using cubic restricted splines of fructosamine and fasting glucose respectively and diurnal age as the underlying timescale. Hence, we depicted a continuous risk curve of HRs with 95% CI over the complete range of respective marker. Further, the lowest ordered 10% of fructosamine was compared to the reference group and hazard ratios with 95% CI were estimated for all study outcomes.

Study IV

Participants

All individuals who had a fasting glucose measurement in the baseline period (1985-1996) were included in the study population except for those with a diabetes diagnosis at baseline, either documented, self-reported or diagnosed by the CALAB fasting blood glucose (≥ 7.0 mmol/L) (n=296,439).

Metabolic risk factors

Potential risk factors investigated in this study included glucose, fructosamine, haptoglobin, uric acid, triglycerides, total cholesterol, BMI, apoB, apoA-I and the apoB/apoA-I ratio.

Outcome and follow-up

The follow-up continued from the baseline examination until a diagnosis of T2D (the event of interest), emigration, death or June 30th 2012. The diagnoses were obtained from the national diabetes register, the national patient register, the national prescribed drug

register, the CALAB repeated blood samples and self-reports. The earliest record from any of those registers constituted type of diagnosis and year of diagnosis in the analysis.

Nested case-control sample

To facilitate the analyses of trajectories, we constructed a nested case-control sample from the study population. New cases of T2D during the follow-up period were matched to five controls by sex, age group and calendar time in an incidence density sampling approach.¹³⁹ Number of years (NoY) from the first measurement of fasting glucose and the diagnosis/control selection was calculated.

Statistical analysis

In the study population, hazard ratios accounting for one standard deviation of respective risk factor (95% CI) were estimated with Cox PH regression.¹³⁸ In addition, hyperglycemic cut-offs for fructosamine were set and hazard ratios estimated. We estimated absolute 10- and 20-year risks stratified on sex, age, BMI, triglycerides and glucose through logistic regression models. Weighted mean values and 95% CI were calculated for all risk markers by each NoY and trajectories were described graphically for respective factor. We used the distribution of sex, age group and calendar time of the full nested case-control sample as reference population in the weighting procedure.

RESULTS

STUDY I

Subject characteristics

The study included 10,987 subjects, whereof 47% were males, with a wide range of glycemic exposure. Out of those, 7,591 had normal glucose tolerance (n=5,714) or were in a pre-diabetes state (n=1,877). Subjects with T2D were on average 20 years older

compared to subjects with T1D (Table 5). For either newly diagnosed T2D or previously diagnosed T2D, the mean levels of fructosamine, serum glucose and HbA1c were similar in both fasting and non-fasting subjects. Subjects with T1D had on average higher levels of fructosamine, HbA1c and serum glucose.

Table 5. Characteristics of individuals with diabetes in the study population (Study I)

	NewT2D		DiagnosedT2D		T1D	
	Fasting	Non-fast	Fasting	Non-fast	Fasting	Non-fast
N	759	497	825	1,035	88	192
% of pop	7%	5%	8%	9%	1%	2%
Female sex	33%	35%	37%	41%	44%	46%
Age (mean)	61 (12)	62 (12)	60(11)	62 (11)	41 (11)	40 (13)
Age (years)						
-30	0	0	1	1	19	46
30-49	131	73	151	155	47	93
50-69	432	273	514	600	22	52
70-	196	151	159	279	0	1
BMI	29 (4.7)	29 (5.3)	29 (5.0)	28 (5.0)	26 (4.6)	26 (4.3)
Fructosamine	2.62 (0.52)	2.83 (0.54)	2.71 (0.58)	2.77 (0.55)	2.93 (0.62)	3.11 (0.63)
HbA1c	6.93 (1.66)	7.92 (1.53)	7.21 (1.77)	7.41 (1.70)	7.62 (1.58)	7.88 (1.56)
Glucose	9.55 (3.01)	11.1 (4.20)	9.61 (3.60)	10.5 (4.60)	11.3 (5.33)	10.5 (5.73)
Albumin	42.9 (2.6)	42.7 (2.9)	42.7 (3.1)	42.8 (2.9)	42.3 (3.4)	42.9 (3.2)
Triglycerides	2.30 (1.80)	2.56 (2.43)	2.32 (2.30)	2.24 (1.98)	1.61 (1.31)	1.27 (0.91)
Total cholesterol	6.07 (1.20)	5.98 (1.26)	6.01 (1.47)	5.96 (1.37)	5.45 (1.32)	5.42 (1.48)
LDL-C	3.83 (1.15)	3.80 (1.07)	3.71 (1.14)	3.71 (1.13)	3.36 (1.28)	3.31 (1.15)
HDL-C	1.27 (0.44)	1.23 (0.39)	1.32 (0.45)	1.30 (0.40)	1.58 (0.58)	1.60 (0.43)
ApoB	1.40 (0.41)	1.40 (0.48)	1.39 (0.48)	1.31 (0.42)	1.27 (0.61)	1.04 (0.37)
ApoA-I	1.38 (0.23)	1.38 (0.24)	1.39 (0.24)	1.38 (0.21)	1.46 (0.26)	1.46 (0.23)
ApoB/ApoA	1.04 (0.34)	1.03 (0.31)	1.03 (0.40)	0.97 (0.31)	0.88 (0.41)	0.73 (0.27)
Creatinine	85.7 (16.3)	86.7 (19.0)	86.4 (19.7)	89.4 (35.7)	85.2 (23.8)	82.2 (18.7)
eGFR	79.3 (16.3)	77.5 (17.2)	79.3 (17.1)	76.0 (19.4)	91.8 (20.3)	92.4 (17.8)
Education	35%	35%	37%	36%	20%	21%
Sweden born	79%	76%	77%	81%	86%	90%
B-C, Workers	14%	13%	18%	17%	19%	17%
History, CVD	7%	6%	13%	14%	7%	3%
History, cancer	7%	8%	7%	8%	2%	3%
CKD	14%	16%	12%	21%	9%	7%
Anemia	0.9%	0.2%	1.8%	2.5%	2.3%	1.0%

Cross-sectional correlations between metabolic biomarkers

The linear correlation between fructosamine and HbA1c in fasting subjects was $r=0.75$, $r=0.75$ and $r=0.67$ in NewT2D, DiagT2D and T1D respectively (Figure 4). In non-fasting subjects with T2D, fructosamine and HbA1c correlated somewhat less. Adding a non-linear component in the correlation analysis increased the correlations marginally for DiagT2D ($r=0.76$) but not for NewT2D. The correlation between fructosamine and HbA1c

was much lower in subjects with prediabetes ($r=0.11$) and was null in subjects with normal glucose tolerance (not shown). In comparison, fructosamine correlated higher to total cholesterol ($r=0.31$, $p<0.001$), triglycerides ($r=0.18$, $p<0.001$) and albumin ($r=0.23$, $p<0.001$) in subjects with normal glucose tolerance compared to fasting glucose ($r=0.12$, $p<0.001$) and HbA1c ($r=-0.01$) respectively (post publication analysis).

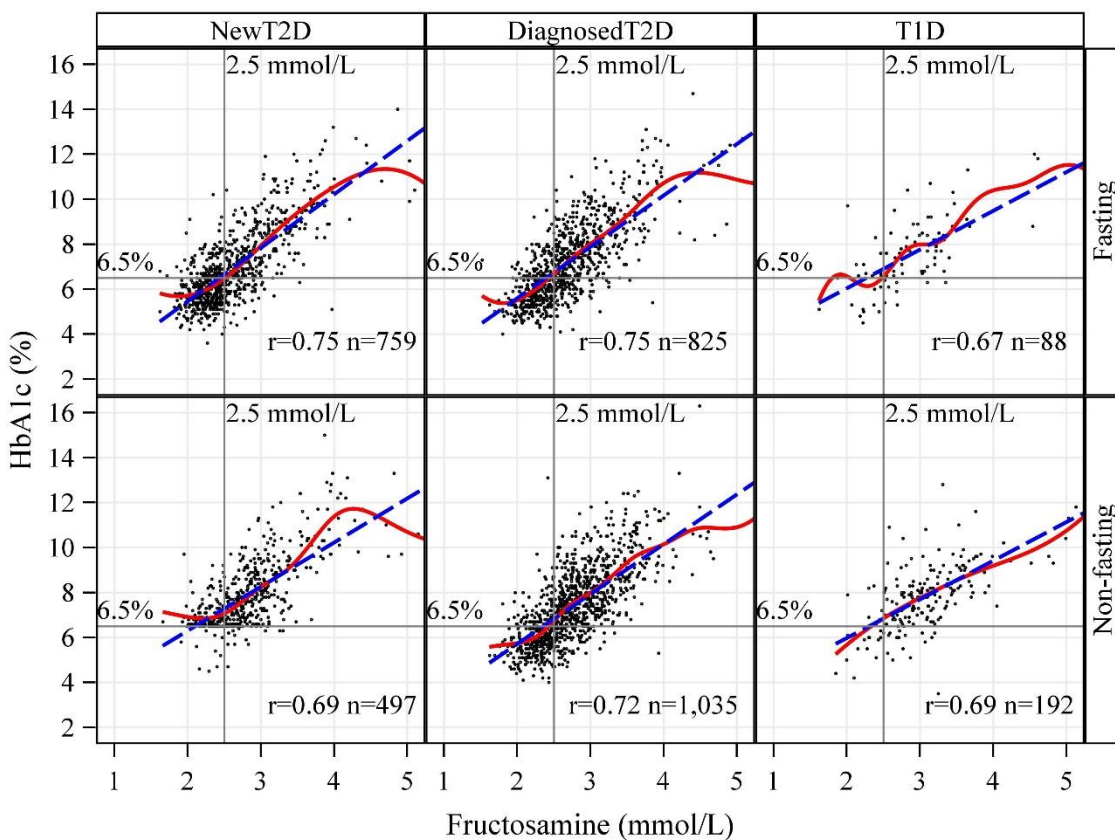


Figure 4. Scatterplots of fructosamine and HbA1c by type of diabetes and fasting status. Pearson linear correlation coefficients (r), linear regression curve (blue dashed line) and smoothed penalized splines (solid red line) are indicated in respective graphs. Diagnostic diabetes cut-off for HbA1c (6.5%) according to guidelines and potential cut-off for fructosamine (2.5 mmol/L) are indicated.

Longitudinal analysis of changes in simultaneously measured biomarkers

To evaluate the correlation between the three measurements of glycemic exposure over time, we identified and restricted to those subjects who had simultaneous measurements of all three biomarkers (fructosamine, HbA1c and glucose) at an index examination and in two consecutive time windows during one year. For those who had increased their

glucose after an average of 290 days, fructosamine and HbA1c had increased in parallel (Figure 5). Correspondingly, for those who had decreased their glucose levels after 290 days, also had decreased their levels of fructosamine and HbA1c respectively. Newly diagnosed as well as long-standing T2D were similar in this development.

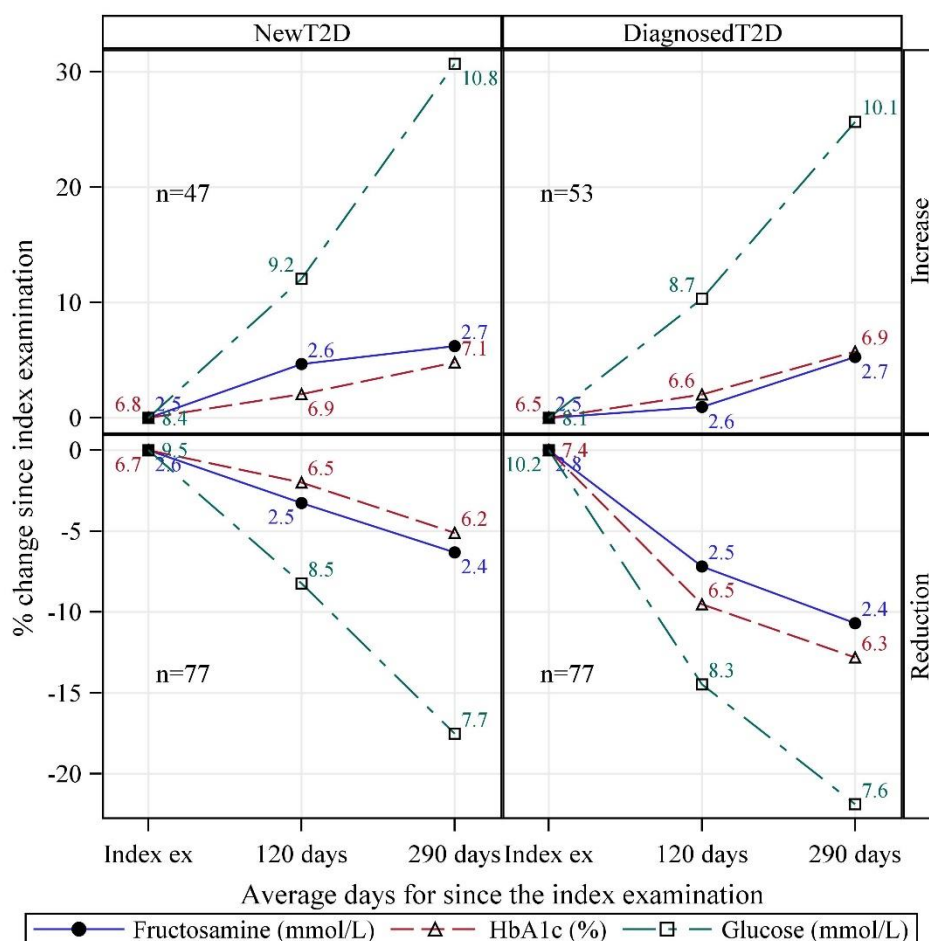


Figure 5. Mean values of fructosamine, HbA1c and glucose at the index examination and within two time windows in the following year.

STUDY II

Study participants' characteristics

The study included 338,443 subjects. Men (53%) and women were about 48-50 years on average at baseline, i.e. the time of the first blood sampling. About 60% were fasting at the baseline measurement (Table 6). On average, men had higher fructosamine compared to women, 2.13 vs. 2.05 mmol/L (not shown). Consequently, proportionally more men than women were in the higher fructosamine categories (Table 6). Furthermore, subjects with elevated fructosamine were older on average

compared to subjects with lower levels of fructosamine. Triglycerides and glucose were positively associated with the fructosamine categories. Total cholesterol was similar in all groups above 2.30 mmol/L.

Event rates of MI and death

We observed 21,526 incident MIs and 73,458 deaths during the follow-up. The event rate of MI was higher among men (44 cases/10,000 person years) than among women (24 cases/10,000 person years). The sex difference in mortality was less pronounced than that observed for incidence of MI.

Table 6. Subject characteristics at first visit – stratified by fructosamine levels.

	Fructosamine levels (mmol/L)				
	<1.78	1.78-2.29	2.30-2.59	2.60-2.69	≥2.70
n, subjects	18,371	271,832	39,246	2,335	5,305
% with repeated measures	43%	45%	37%	43%	54%
Female	64%	48%	35%	33%	31%
Fasting (%)	60%	58%	60%	61%	58%
Age inclusion, years (range)	47 (39-54)	49 (40-56)	52 (42-60)	55 (46-64)	58 (49-65)
Socioeconomic status					
Low	49%	43%	37%	39%	39%
High	44%	50%	53%	47%	44%
Not gainfully employed	7%	7%	10%	14%	17%
Fructosamine (mmol/L)	1.7 (0.1)	2.1 (0.1)	2.4 (0.1)	2.6 (0.0)	3.2 (0.5)
Glucose (mmol/L)	4.7 (0.7)	4.9 (0.7)	5.2 (1.2)	6.4 (2.2)	11.0 (4.8)
Total cholesterol (mmol/L)	5.2 (1.0)	5.7 (1.1)	6.3 (1.2)	6.5 (1.4)	6.3 (1.6)
Triglycerides (mmol/L)	1.2 (0.8)	1.3 (0.9)	1.7 (1.4)	2.4 (2.4)	2.9 (3.4)
Albumin (g/L)	40.8 (3.0)	42.8 (2.7)	43.7 (2.8)	43.3 (3.1)	42.4 (3.1)

Association of fructosamine and incident MI and all-cause mortality

There was a clear positive association of fructosamine levels and incidence of MI (Figure 6) as well as with all-cause mortality (Figure 7). In the model adjusted for sex, age, fasting status and calendar time there was a slightly increased hazard for MI in the prediabetes group compared to the reference group and the hazard ratio increased with higher fructosamine and was highest in the poorly controlled diabetes group (HR=2.9, 95% CI 2.7-3.1). Further adjustment for potential confounders of the association

between fructosamine and MI incidence (i.e. total cholesterol, triglycerides, albumin and glucose) retained the hazard ratio increased (Figure 6). For all-cause mortality we noted the same risk pattern as was noted for incident MI, although the magnitude of the HRs was less (Model 1 HR= 2.3 (95% CI 2.2-2.4)) (Figure 7). Furthermore, there was an increased HR for all-cause mortality when comparing 'prediabetes' and 'Low5%' respectively with the referents even when glucose was included in the multivariate models.

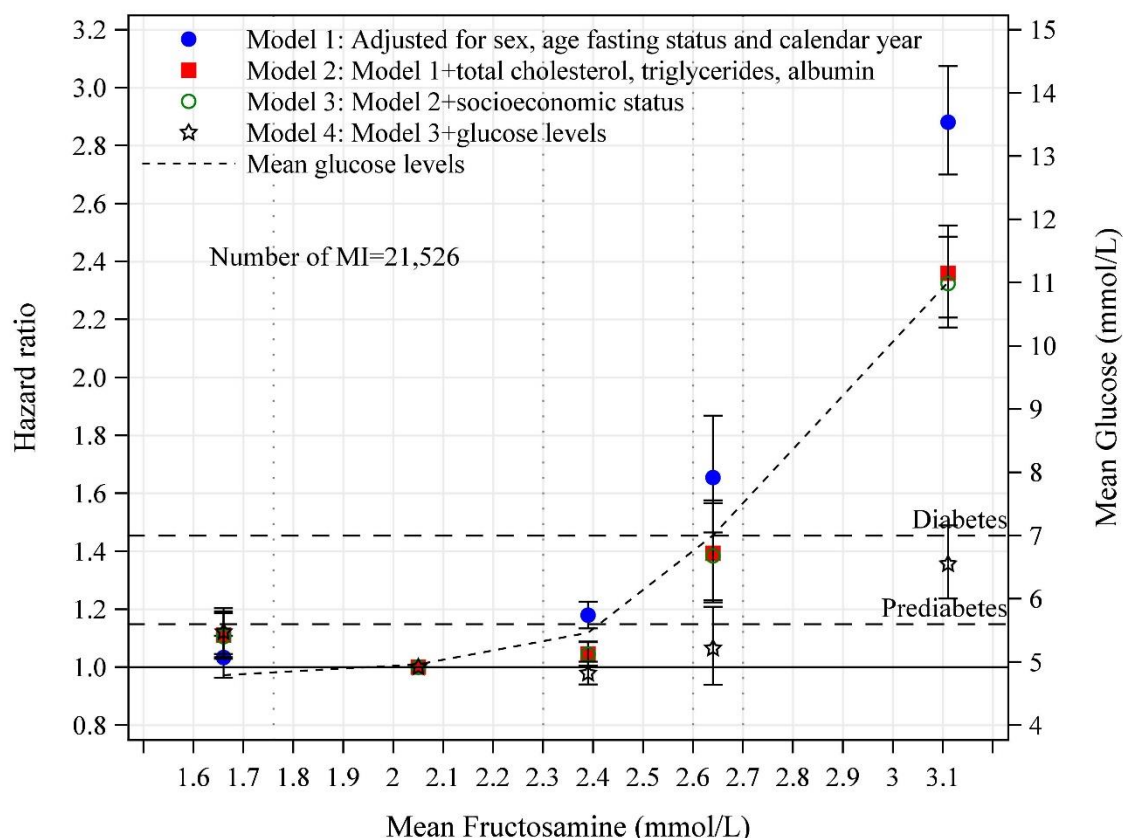


Figure 6. Incident Myocardial Infarction and CHD death, hazard ratios with 95% CI comparing fructosamine in clinical important groups to normal range One subject eligible for counting in multiple fructosamine categories. Vertical dotted lines indicate boundaries for the categories of fructosamine.

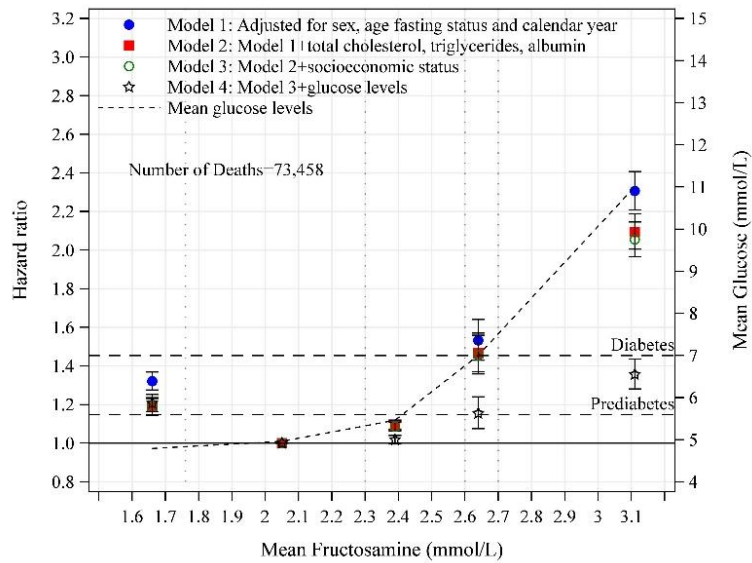


Figure 7. All-cause mortality, hazard ratios with 95% confidence intervals comparing fructosamine in clinical important groups to normal range. One subject eligible for counting in multiple fructosamine categories. Vertical dotted lines indicate boundaries for the categories of fructosamine.

Comparative analysis of fructosamine, HbA1c and fasting glucose regarding the association to MI and death

In a subset of the study population, HbA1c was measured ($n=9,746$) and we categorized those subjects with respect to glycemic exposure. Fructosamine and HbA1c were similar in risk predictions for MI and all-cause mortality in prediabetes, controlled T2D and poorly controlled T2D respectively (Figure 8). There was an indication of higher mortality in the prediabetes group estimated by fructosamine compared to when estimated by HbA1c. In the prediabetes group, the mean level was 2.43 mmol/L and 6.01% for fructosamine and HbA1c respectively. Fasting glucose demonstrated constantly lower HRs as compared to fructosamine and HbA1c. The increased HRs for all-cause mortality were found for fructosamine even after adjustment for HbA1c.

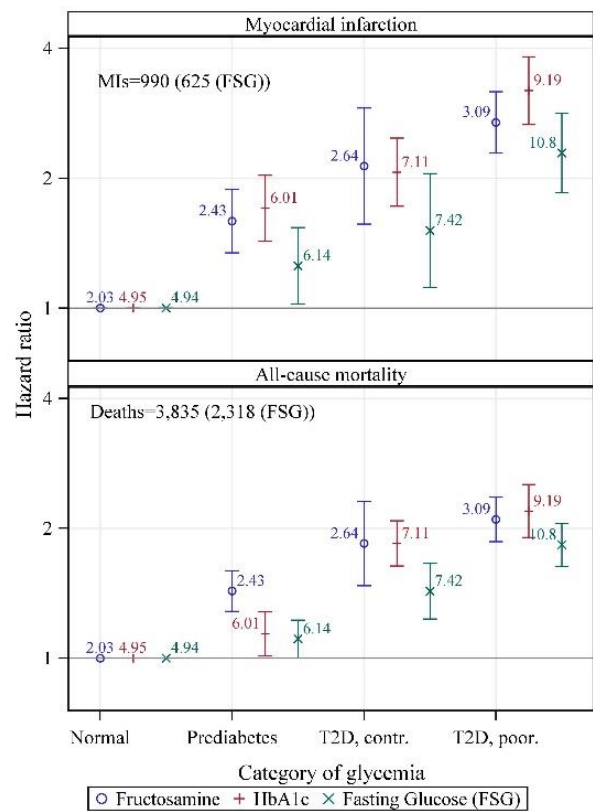


Figure 8. Hazard ratios for MI and all-cause mortality in categories of fructosamine, HbA1c and fasting glucose respectively.

STUDY III

Study participants' characteristics

There were 215,011 individuals included in the study and about half of these were women. Individuals characterized by low fructosamine were younger on average compared to individuals in the reference group (Table 7). In addition, the proportion with low socioeconomic status was higher.

The intersection of low fructosamine and low glucose was 15%. Biomarkers of lipid related exposure (including apo-B and apo-A-I (not shown)) indicated healthier levels in the lowest fructosamine group compared to medium and higher values. Serum haptoglobin was higher in the low fructosamine group compared to the groups with medium or high levels.

Table 7. Subject characteristics of 215,011 individuals with fasting measurements of biomarkers presented in total and by distribution of baseline fructosamine (<10%, 10-90% and >90%).

	Baseline fructosamine			
	Low 10%	Medium	High 10%	ALL
Subjects, n	21,377	173,153	20,481	215,011
Women (%)	47%	47%	47%	47%
Age at baseline (sd)	43 (14)	46 (14)	50 (14)	46 (14)
Low socioeconomic status	47%	43%	39%	43%
Low 10% Glucose (%)	15%	11%	8%	11%
High 10% Glucose (%)	5%	8%	16%	8%
S-Fructosamine (mmol/L)	1.72 (0.1)	2.07 (0.1)	2.40 (0.1)	2.07 (0.2)
S-Glucose (mmol/L)	4.66 (0.6)	4.77 (0.6)	4.95 (0.7)	4.77 (0.6)
Triglycerides (mmol/L)	1.11 (0.7)	1.19 (0.8)	1.49 (1.2)	1.21 (0.8)
Total Cholesterol (mmol/L)	5.08 (1.1)	5.59 (1.1)	6.28 (1.3)	5.60 (1.1)
Albumin (g/L)	41.2 (3.0)	42.6 (2.7)	43.5 (2.8)	42.6 (2.8)
S-Uric acid (μmol/L)	276 (67.3)	286 (69.3)	304 (75.8)	287 (70.0)
Creatinine (μmol/L)	78.4 (13.7)	81.1 (14.2)	84.1 (15.4)	81.2 (14.4)
S-Haptoglobin (g/L)	1.12 (0.4)	1.05 (0.3)	1.01 (0.3)	1.05 (0.3)
Ever smokers (%)^a	36%	27%	21%	27%

^a in a subset of 43,313 subjects (93% women)

All-cause mortality across the fructosamine continuum

The mortality was lowest in subjects with fructosamine levels of 2.20-2.30 mmol/L (Figure 9). Low and intermediate levels of fructosamine were associated with increased mortality. After adjusting for a set of potential confounders, including haptoglobin, the increased mortality at lower fructosamine levels was attenuated. Haptoglobin unaided, accounted for a major part of this reduction in hazard ratios.

Low fructosamine and Cause specific mortality

The cause-specific mortality, i.e. cardiovascular-, cancer-, or all other reasons (not including infection) increased in the low fructosamine group vs. the medium fructosamine group (Table 8). In particular, mortality in lung cancer or chronic obstructive pulmonary disease (COPD) was significantly increased in those with low fructosamine (HR=1.42 (95% CI 1.28-1.58) in adjusted model 2). The major causes of death were otherwise proportionally comparable regardless of fructosamine category.

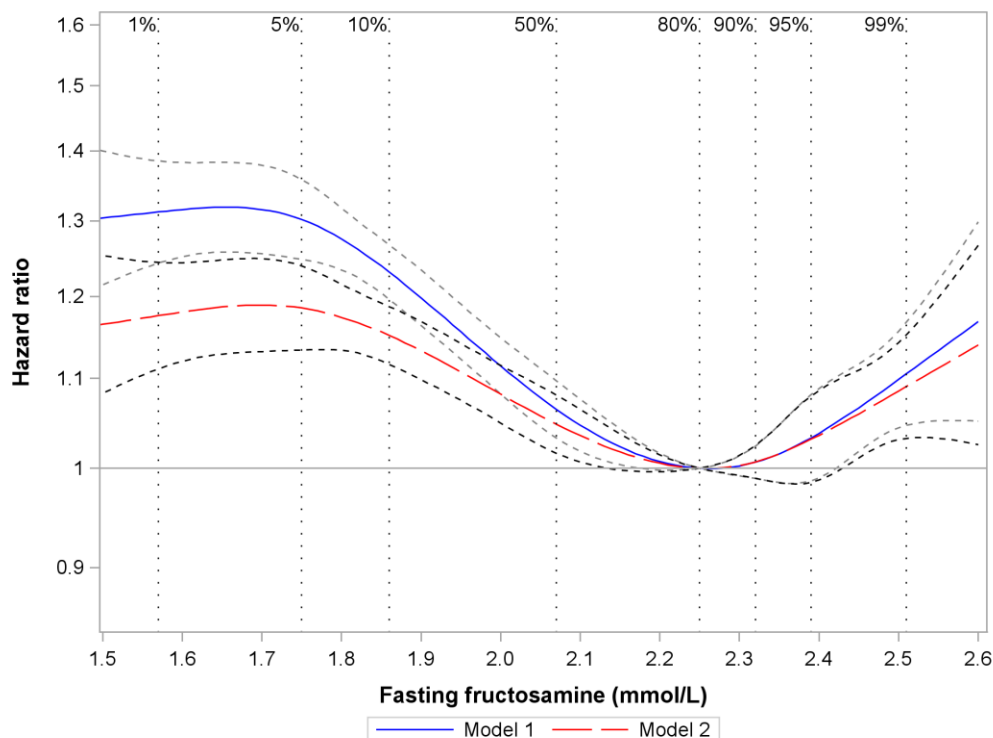


Figure 9. Hazard ratios for all-cause mortality across levels of fasting fructosamine. Model 1 is adjusted for sex, age, social class and calendar period. Model 2 additionally adjusted for total cholesterol, triglycerides, albumin, creatinine, uric acid and haptoglobin. Proportions of subjects concerned are displayed by vertical dotted lines.

Table 8. Hazard ratios for subjects with low fructosamine (1st decile) vs referent fructosamine (medium 10-90%) by cause of death categories.

Cause of death	No. of deaths [†]	Model 1	Model 2
		Hazard ratios (95% confidence interval)	
CVD	15,363 (1,413)	1.08 (1.02-1.15)	1.06 (0.99-1.12)
Cancer	13,781 (1,557)	1.29 (1.21-1.36)	1.15 (1.09-1.22)
Infections	1,341 (129)	1.15 (0.96-1.38)	0.99 (0.82-1.20)
Lung cancer/COPD	3,566 (522)	1.70 (1.55-1.86)	1.42 (1.28-1.58)
All other causes	9,790 (1,037)	1.21 (1.13-1.29)	1.12 (1.04-1.20)
All causes	41,388(4,281)	1.20 (1.16-1.24)	1.11 (1.07-1.15)

Model 1: sex, age, socioeconomic status and calendar period; Model 2: Model 1 + triglycerides, total cholesterol, albumin, uric acid, creatinine and haptoglobin; † Number of deaths (n of deaths in lowest fructosamine decile)

Influence of smoking and inflammation in the relation between low fructosamine and mortality

In sensitivity analyses (not shown), we observed a reduction of the hazard ratio for all-cause mortality in the lowest 10% of fructosamine vs. the reference group when we adjusted for smoking status (ever/never). The reduction extended to almost 25%. In a haptoglobin stratified analysis, we showed similar fructosamine related hazard ratios across all quartiles of haptoglobin in model 1

and none or minor reduction of those hazard ratios when adjusted for haptoglobin.

Additional sensitivity analyses were performed to- a) adjust for any reverse causation from cancer b) adjust for any influence regarding body mass index and c) adjust for an extensive set of biomarkers including apolipoproteins, sodium, potassium and liver related markers. None of these analyses substantially differed from the results of the main analyses.

STUDY IV

Study participant characteristics

There were 296,436 individuals included in the study population. On average, the study subjects were 44.8 years at the baseline examination, all were fasting and 47% of them were women.

Characteristics of T2D cases

The mean age of a T2D diagnosis was 63.2 years. The T2D cases were more frequently of low socioeconomic status compared to controls. Furthermore, the T2D cases had higher BMI and higher levels of most of the biomarkers related to glycemic, lipid and inflammatory exposures, except for apo A-I, which was lower compared to the controls.

Associations of metabolic risk markers and diagnosis of T2D

Markers of glycemic, lipid and inflammatory exposure respectively were all associated with the incidence of T2D, in sex, age and calendar time adjusted models. Most markers showed an association after adjustments for triglycerides, glucose and uric acid respectively. The risk of T2D was increased in two defined categories of pre-diagnostic fructosamine after adjustment for total cholesterol, fasting triglycerides and fasting glucose. The adjusted risk was 33% higher

for those with fasting fructosamine between 2.46 and 2.59 mmol/L compared to those below 2.33 mmol/L (Table 9).

Trajectories describing the development of metabolic markers before T2D

We identified 28,235 new cases of T2D during the follow-up period. In graphically described trajectories, mean values of several biomarkers were increased in T2D subjects vs. controls more than 20 years before the diagnosis/control selection. BMI, fasting glucose and triglycerides were below clinical cut-offs for obesity, hyperglycemia and hypertriglyceridemia respectively, at 20 years before diagnosis, yet at higher levels compared to controls (Figure 10).

Fructosamine was different in T2D cases compared to controls at 15 years before the diagnosis/control selection and this difference increased closer to diagnosis of the cases. In addition, other markers including uric acid, haptoglobin and apoB/apoA-I ratio were increased in cases compared to controls long before the diagnosis. Accelerated differences between cases and controls over time, primarily appeared for glucose and fructosamine, while more constant differences were observed for triglycerides, BMI, uric acid and haptoglobin.

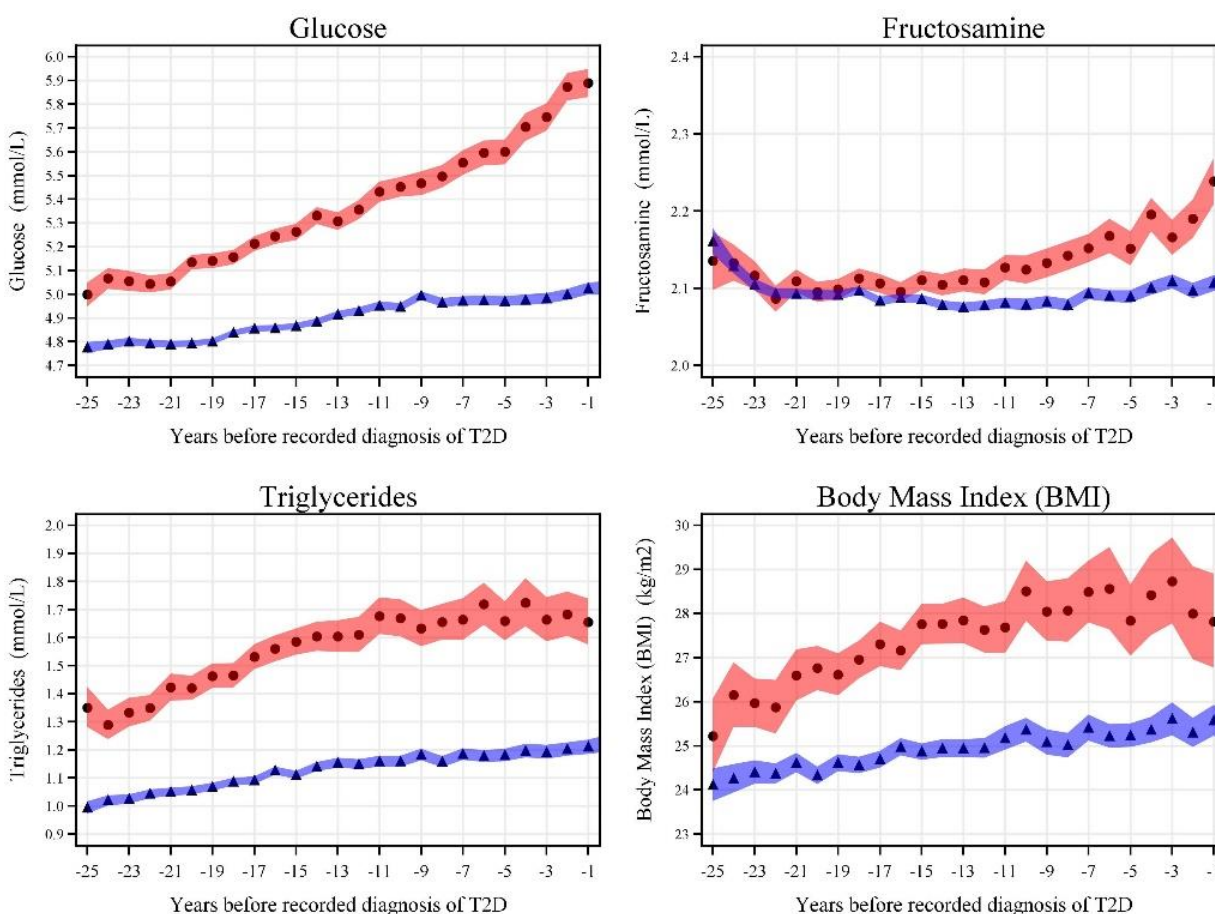


Figure 10. 25-year trajectories of fasting glucose, fructosamine, triglycerides and BMI in T2D cases and controls respectively.

Prediction of long-term risk of T2D

The observed 20-year risk of T2D was 8.1% in the study population. In both men and women, the risk increased considerably with higher BMI (Figure 11). Elevated blood lipids in the form of triglycerides ≥ 1.4 mmol/L (124 mg/dL) approximately doubled up the risk of T2D independently of BMI. With a BMI of

≥ 30 kg/m², triglycerides ≥ 1.4 mmol/L and a fasting glucose ≥ 5.6 mmol/L (100 mg/dL) the estimated 20-year risk of T2D was 64% and 70% in men and women aged 40-49 years respectively. The risk remained high in those individuals even at normal glucose levels and was 21% and 18% in men and women respectively.

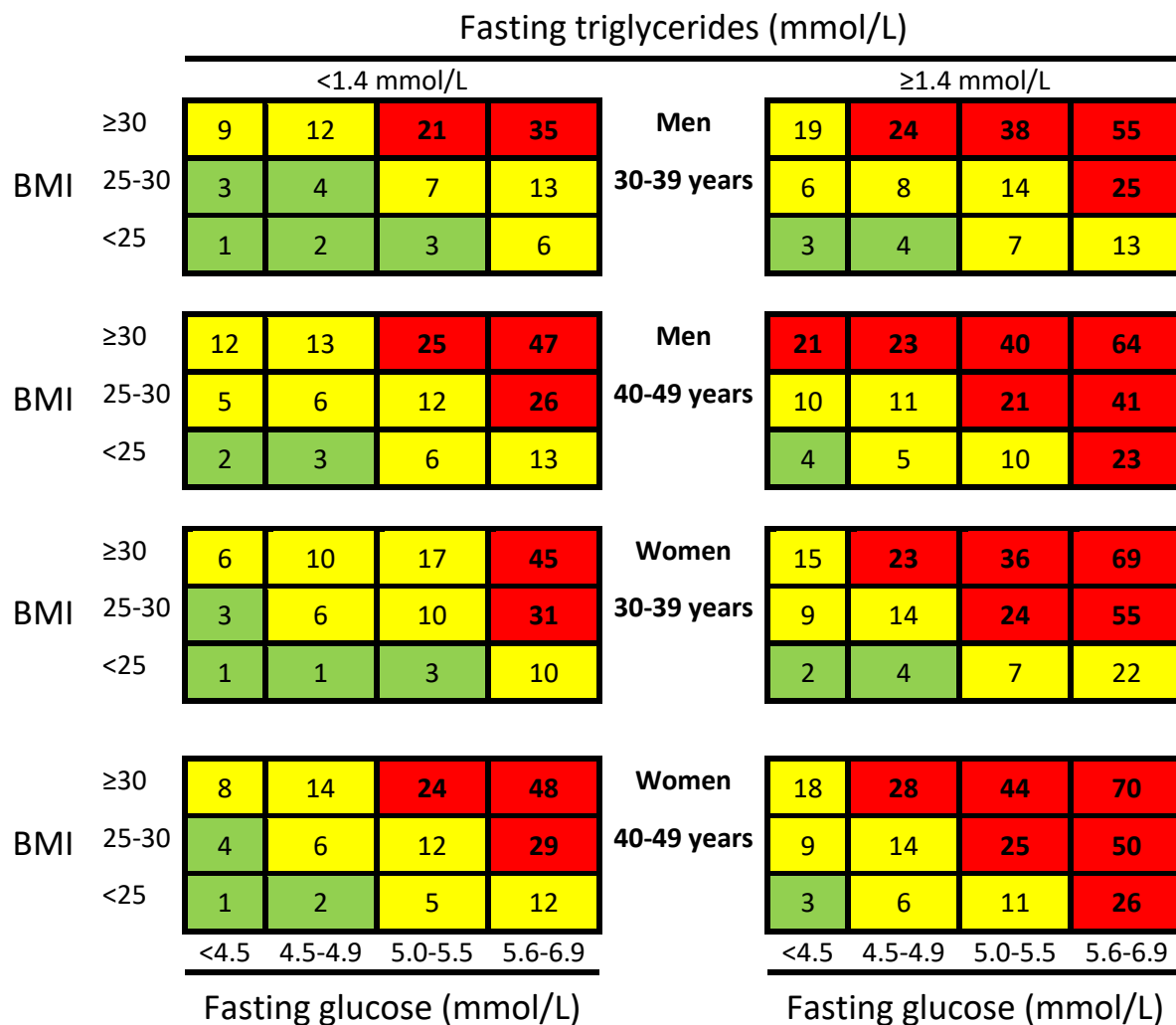


Figure 11. Estimated 20-year absolute risk of T2D based on sex, age, BMI, fasting glucose and triglycerides. Green color (risk <5%), yellow color (risk 5–20%), red color (risk ≥ 20%). BMI: kg/m².

Table 9. Adjusted hazard ratios (95% CI) of baseline fasting fructosamine and incident T2D. Categories correspond to < FSG 5.6 mmol/L, FSG 5.6–6.0 mmol/L, 6.1–7.0 mmol/L and AMORIS diabetes cut-off for fructosamine. Based on 202,183 subjects and 18,285 incident T2D cases.

Fructosamine (mmol/L)	Model 1 ^a	Model 2 ^b	Model 3 ^c
<2.33	1.00 (reference)	1.00 (reference)	1.00 (reference)
2.33-2.45	1.35 (1.28-1.42)	1.20 (1.14-1.26)	1.08 (1.02-1.13)
2.46-2.59	1.77 (1.64-1.91)	1.33 (1.23-1.44)	1.16 (1.08-1.26)
≥2.60	2.45 (2.20-2.74)	1.38 (1.23-1.54)	1.23 (1.10-1.38)

^a adjusted for sex and age; ^b adjusted for sex, age, triglycerides, total cholesterol; ^c adjusted for sex, age, triglycerides, total cholesterol and fasting glucose

DISCUSSION

MAIN FINDINGS

In a large population, we have demonstrated a strong linear association concerning measurements of fructosamine and the major glycemic determining biomarkers glucose and HbA1c. We showed a curvilinear correlation in those measurements, where states of hyperglycemia or diagnosed diabetes of either type 1 or type 2 constituted thresholds. As expected, changes in glucose over time were reflected in similar parallel increases or decreases in fructosamine and HbA1c. Fructosamine levels may differentiate subjects with and without hyperglycemia. Measurements of fructosamine correlates well with glucose levels in the hyperglycemic range and has the clinical advantage that fasting is not a prerequisite to obtain reliable values.

Elevated levels of fructosamine were associated with the incidence of acute myocardial infarction and mortality in the total study population. The excess risks for these outcomes were largely the same for either fructosamine, fasting glucose or HbA1c. One would presume that the molecular mechanism that causes damage would be the same or similar for all these three markers and such a mechanism have been discussed previously. The molecular mechanisms underlying the mortality-increasing associations of low levels of fructosamine, also pointed out previously,¹⁰⁵ remain to be explained in detail, yet some of

these associations could be due to concomitant smoking and inflammatory mechanisms.¹⁴⁰⁻¹⁴³

Lastly, up to 25 years prior to diagnoses, individuals who developed T2D differed from those who did not with regard to several factors, among them fasting glucose, triglycerides and BMI. A difference in fructosamine between T2D cases and controls appeared more closely to the diabetes diagnosis than when a difference in fasting glucose was observed. Thus, our results suggest that increased fructosamine compared to a reference population may appear about 15 years before the diagnosis of T2D. The increased risk of elevated fructosamine at pre-diagnostic levels was low as compared to glucose, triglycerides and BMI, yet present and of importance as a marker of forthcoming risk. Altogether, these findings of higher values of fasting glucose, fructosamine, triglycerides and BMI in cases compared to controls may raise consideration about when and why T2D starts to develop. The major findings of this thesis may substantiate the utility of fructosamine as a glycemic determinant in clinical practice and epidemiological research.

METHODOLOGICAL REFLECTIONS

Selection bias

If the selection process of individuals into the study is associated with the outcome and/or the exposure, bias may arise.¹⁴⁴ Then the exposure-outcome relationship may no longer be representative to that of the target population from which the study population arose.¹⁴⁵

Fructosamine, the main exposure of interest in this thesis, was included in a standard package of analyses that was a no-fee test offered by the CALAB laboratory. Hence, any suspicions of disease is not likely to influence the recording of fructosamine and the selection of subjects into the different studies of this thesis. Thus, we assume that the selection process into the studies should have been similar for cases and non-cases (Study II-IV).

Any loss-to follow-up affected by exposure or disease could bias the epidemiological measures. By use of national event registers (Study II-IV) and emigration register, there should be a very small loss to follow-up in the studies of this thesis.

The AMORIS cohort had lower mortality compared to the population of Stockholm County during the period 1985-2012 because of a predisposition of gainfully employed subjects (Standardized Mortality Ratio=0.88).¹²⁴ By suffering from chronic illness, the chance to be employed is lower.¹⁴⁶ The absolute incidence rate of myocardial infarction and mortality rate in study II and

study III would be expected to be lower compared to that of the population of the greater Stockholm area. However, it is unlikely that this would bias the associations between fructosamine levels and these outcomes.

Selection of individuals with available information could give a selection bias if event-related sources such as quality of care registers are used. This may lead to a higher probability for the cases to be exposed. In attempting to avoid potential selection bias, in study II and III, we restricted our sensitivity analyses to those who had information on smoking from research cohorts, the MFR or national surveys of living conditions exclusively. This restriction limited the sample size.

Confounding

In randomized clinical trials, risk factors other than that under study theoretically should be of equal distribution in the randomized groups. In contrast, the observational design with non-randomized exposure groups inevitable suffers from oblique distributions. Confounding, is a population phenomenon and is present when an extraneous risk factor for the disease under study also is associated with-, but not affected by-, the exposure of interest. Several methods are available for controlling confounding.¹⁴⁷ By using these methods, one attempts to reduce the effect on the studied event relation that is associated with the potential confounders and eventually claim the exposure effect as independent of other risk factors. Confounding will

frequently remain and result in “residual confounding”. The magnitude of residual confounding may depend on several factors, including misclassification and categorization of variables.¹⁴⁷ Furthermore, researchers have almost never exhaustive access to information necessary to claim causality.

In the present studies, we had information on biochemistry markers that potentially could present as confounders to any observed association between fructosamine and the outcomes. By adjusting for a set of relevant biochemistry markers, we aimed at reducing confounding from those factors. Generally, we used the continuous measurement of those markers. In all studies, we had access to data on occupational activity from national censuses and we classified groups of socioeconomic status (SES) based on this information. For many medical associations, SES may be an important confounder.¹⁴⁸ Regarding other major risk factors for cardiovascular disease and mortality such as smoking and hypertension, we had limited information. Yet, in a sensitivity analysis, we did not see any major changes in the risk estimates when we adjusted for these variables (Study II). In a previous study, current smoking had no major association with hyperfructosemia.¹⁰⁶ Hence, per definition smoking did not constitute an actual confounder on the event relation of high fructosamine and cardiovascular disease and mortality respectively. On the other hand, smoking has been associated with lower fructosamine.¹⁴⁰ Again, in study III, we had limited data on smoking. However, we were

able to adjust in a subset and smoking inferred a change in the risk estimate towards the null.

Information bias

Misclassification of disease

In studies II-IV, we obtained all disease information and mortality from high quality national health and mortality registers. These registers, covering census, enable good sensitivity and specificity of outcome but a certain degree of misclassification will be present. Regarding all-cause mortality, there is basically no misclassification. Diagnoses of MI, obtained from the national inpatient register compared to medical records, have been validated and considered being of high quality.^{129,149} The specificity for MI is high. It is clear that not all MIs are reported as such (e.g. silent MIs). Hence, we included in the outcome also subjects deceased in CHD (Study II). Even so, a reduced sensitivity would likely be non-differential as regard to fructosamine exposure, and in theory not affect the risk ratios.¹⁴⁷ Misclassification of T2D could be high in sensitivity terms and low as regards specificity. Compared to the estimated age stratified diabetes prevalence in Sweden 2012,¹⁵⁰ the corresponding numbers as shown in Table 10 for the AMORIS cohort were similar. Furthermore, the incidence rates in AMORIS corresponds well to those estimated nationally (Table 11). Therefore, the rates of T2D diagnoses in study IV is comparable to current Swedish national estimates. This supports the validity of the T2D diagnoses used in the present thesis,

which were obtained using five data sources and primarily the NDR.

Misclassification of exposure

If the exposure of interest is misclassified independently on disease outcome, the effect on the estimated parameter measure (e.g. risk ratio, hazard ratio, odds ratio) is generally predictable in direction, that is, towards the null. In cohort designs, where the exposure is collected at start of follow-up this is often the case.

We used high quality measurements of all biochemistry markers obtained by the CALAB laboratory. Intra-individual and inter-individual variations of fructosamine was considered as minor misclassified with low coefficient of variation (CV%).⁸⁹ Similarly, for exposure variables reported in study IV, the variation was low for either biomarker. Daily variation in fructosamine levels could potentially affect the measurement of interest and misclassify borderline individuals into adjacent exposure categories. We assume that this misclassification would have occurred independently of future disease outcome and hence diluted the observed associations towards no relation. In study II, we categorized fructosamine into multiple exposure categories. Primarily, those categories were defined in consideration to mean levels of HbA1c, where HbA1c started to increase at a fructosamine of 2.30 mmol/L (prediabetes) and exceeded diabetes cut-off (6.5%) at 2.60 mmol/L. The epithets (i.e. prediabetes, well-controlled diabetes and

poorly controlled diabetes) of those categories were arbitrarily given because no established cut-off values for diabetes are set for fructosamine. Still, the relations of the defined ranges of fructosamine and MI/CHD death and mortality should not be afflicted with any major bias. Because the use of multiple exposure categories, in theory, the predicted direction towards the null association could be invalid. However, given the observed dose-response relationship of fructosamine and outcome the potential bias seems to be non-differential (Study II).

Misclassification of confounders

In all four studies, we adjusted our estimates for potential confounders. Apart from sex and age, we primarily adjusted the models for other biochemical markers and for these we do not believe that there is any major misclassification regarding baseline values. Furthermore, the use of continuous scale for those markers, avoids any potential categorization issues.¹⁴⁷ In study II, we adjusted for smoking and hypertension in sensitivity analyses. Information on those factors came predominantly from questionnaire data and hence a certain degree of misclassification most likely exists. Any misclassification of potential confounders would limit the possibility for valid adjustment for the confounding effect of the estimated parameter.¹⁴⁷ Importantly, we used only information collected before the start of follow-up and that would reduce any differential bias affected by the disease outcome.

Proportional hazards

The assumption of proportional hazards (PH assumption) across time is integral in Cox PH regression. In its essence, it assumes that the rate effect of a covariate is constant between compared groups at each point in time. Only the background rate will vary. We evaluated the assumption of proportional hazards by inspection of the Schoenfeld residuals^{138,151} obtained in the PH modelling, and found their correlations with age at the outcome to be none or very weak. Conservatively we

deemed the assumption to hold for the covariates included in the fitted models (Study II-IV). There were signs of a departure from proportionality in the hazard ratio comparing men and women thus indicating a conjunction between men and women at older ages regarding the risk of MI, CHD and T2D. We stratified our models by sex rather than included it as a covariate. Stratification or inclusion of time dependent covariates are methods to consider when the PH assumption tends to be severely violated.

Table 10. Prevalence of T2D in 1996, 2004 and 2012 in the AMORIS population.

Prevalence (%) of T2D at three calendar years							
		1996		2004		2012	
Age (years)	Sex	Mean age	Prevalence	Mean age	Prevalence	Mean age	Prevalence
35-44	Men	39.6	1.1%	40.0	0.9%	41.3	1.2%
	Women	39.6	0.7%	39.8	0.6%	41.2	1.4%
45-64	Men	53.6	5.6%	55.2	6.8%	55.3	6.9%
	Women	53.6	2.8%	55.4	3.7%	55.1	4.1%
≥65	Men	74.3	8.9%	73.4	13.8%	73.5	17.8%
	Women	76.3	6.5%	74.9	9.4%	74.6	11.5%
ALL	Men	51.0	4.5%	57.0	7.5%	63.0	11.7%
	Women	51.9	2.8%	57.7	4.8%	63.6	7.5%

Table 11. Incidence rates of T2D (1996-2012) in the AMORIS population.

Incidence rate per 10,000 years in three periods				
Age (years)	Sex	1996-2004	2005-2012	1996-2012
35-44	Men	14.6 (13.5-15.7)	21.5 (19.5-23.5)	16.7 (15.7-17.7)
	Women	9.1 (8.1-10.1)	15.2 (13.5-16.9)	11.2 (10.3-12.1)
45-64	Men	60.5 (59.0-62.0)	84.5 (82.4-86.6)	70.1 (68.9-71.3)
	Women	33.6 (32.4-34.8)	47.8 (46.2-49.6)	39.4 (38.4-40.4)
≥65	Men	101.5 (98.4-104.6)	134.2 (130.9-137.5)	119.1 (116.8-121.4)
	Women	74.1 (71.6-76.6)	93.1 (90.4-95.8)	84.0 (82.1-85.9)
ALL (20-)	Men	53.1 (52.1-54.1)	90.2 (88.6-91.8)	67.9 (67.0-68.8)
	Women	34.7 (33.9-35.5)	58.6 (57.3-59.9)	44.4 (43.7-45.1)

FINDINGS AND IMPLICATIONS

Study I

In this population, including individuals in all stages of glycemic exposure, we showed how three types of glycemic measurements were associated at several exposure levels. We showed substantial correlation in states of hyperglycemia, where the fructosamine vs. HbA1c correlation was $r=0.75$ in both newly diagnosed and documented T2D as well as in T1D. These high linear correlations confirmed previous reports from several studies.¹⁰⁰⁻¹⁰⁴ In stratified analyses, we showed that fructosamine and HbA1c were linearly uncorrelated in subjects that were in a normoglycemic state, consistent with a previous report¹⁰³ of curvilinear correlation, however in a larger population and in another setting. As pointed out,^{106,109} an inverse relationship of fructosamine and BMI potentially could have biased the correlations observed in our study. By adjusting the correlations by BMI, we could not show any major differences. However, this could potentially have implications for the interpretation of fructosamine values in highly obese diabetes patients but this needs to be further studied.

In addition to linear correlations in a cross-sectional setting, we showed high correlations between fructosamine and HbA1c in longitudinally changes. Although this was expected, it has not previously been reported.

With this study, we confirmed previous reports¹⁰⁰⁻¹⁰⁵ and added the largest study performed to investigate the correlations of

the glycemic markers under study. It shows that fructosamine reflects current glucose and HbA1c levels as well as parallel changes over time in states of hyperglycemia. In most situations, fructosamine levels indicate the glycemic exposure similarly as does HbA1c.

Study II

The results from this study suggest an association of elevated fructosamine with incident myocardial infarction as well as with all-cause mortality. An increased incidence of MI was observed already at fructosamine levels indicating prediabetes and the risk of an MI was almost three times higher in individuals with poorly controlled diabetes (as assessed by fructosamine) vs. the reference group. The association remained after adjustment for socioeconomic status, lipids and glucose, which would signify an additional influence of fructosamine above that of lipids and glucose. We used outcomes with comparatively low (MI) or none (all-cause mortality) misclassification and all information on outcomes was obtained from the national patient register and national mortality register. Loss-to follow-up of subjects should not bias the associations. In this study, we categorized fructosamine levels into clinically relevant sub-groups to simplify interpretation of the effects. We also modelled the relationship between fructosamine and mortality continuously rather than in clinically relevant groups and found the sharply increased mortality associated with elevated fructosamine to level-out at around 3.30-3.50 mmol/L. This may indicate that extremely high

fructosamine values could have another underlying mechanism not related to hyperglycemia.

The relation between elevated fructosamine and incidence of hard events such as MI and death was not extensively investigated until 2015. Then, we and two other research groups independently published results on this topic in different populations.^{105,111,152} All these three studies adopted cohort designs and estimated hazard ratios for CHD events and death at various defined cut-offs for fructosamine. In spite of these similarities, direct comparisons of the results are difficult to accomplish mainly due to different population characteristics, choices of reference groups and exposure levels. Still, all studies showed an increased adjusted hazard for a group with higher levels compared to a reference group (Figure 12). The model

adjustments differed between the studies, but all used sex, age and lipid related markers. In addition, one study showed that subjects with well-controlled diabetes had higher hazard of CHD disease compared to the non-diabetes reference group despite of having lower fructosamine.¹⁰⁵ Zaccardi et al. refrained from concluding an independent effect of fructosamine.¹¹¹ The lack of a significant association in this study is most likely due to the smaller study size. It is notable that the mean fructosamine levels were higher in the studies by Selvin et al. and Zaccardi et al. compared to the AMORIS study. In contrast to our study, which utilized fresh blood for all analyses, these other two studies re-analyzed long-term frozen samples. In addition, the different population characteristics may call into questions; what are normal fructosamine levels?

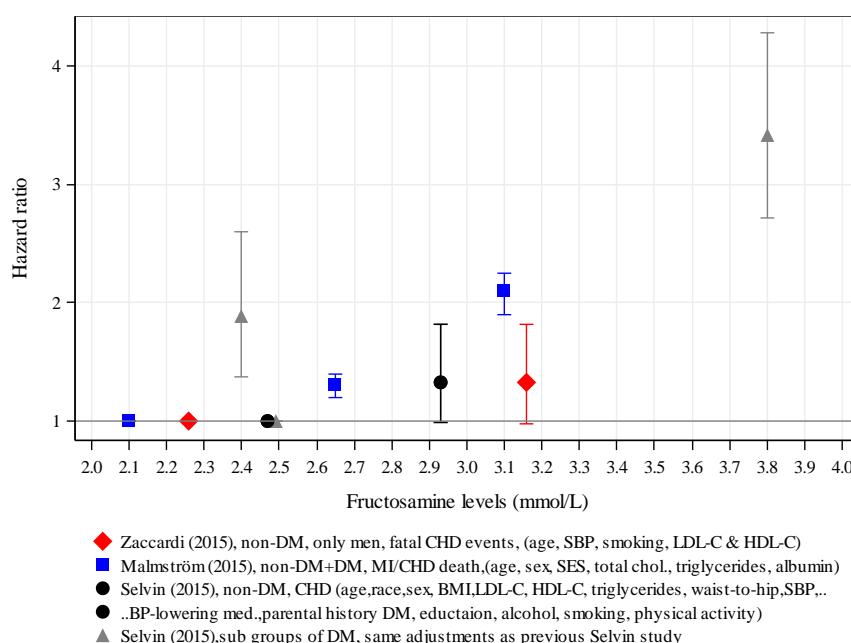


Figure 12. Comparison of three recent studies on the relationship between fructosamine and CVD events.^{105,111,152}

Study III

We included 215,011 subjects without diabetes in this study. All had biochemical measurements, including fructosamine, performed in a fasting state. It has previously been shown how low fasting glucose may be associated with an increased mortality.¹¹² Our main interest in this study was to characterize the mortality across the fructosamine continuum in a population without diabetes, including very low levels. We showed a pattern where all-cause mortality was increased in subjects with low and intermediate fructosamine levels. Those levels concerned up to 80% of the population. Importantly, the pattern of fasting glucose also showed an increased mortality at low levels below 4.4 mmol/L. Several studies have previously shown glucose independent associations between glycated serum proteins, smoking and systemic inflammatory markers.¹⁴⁰⁻¹⁴³ In the ten percent lowest fructosamine, we observed a tendency of increased chronic inflammation (as indicated by increased haptoglobin) and a higher proportion of smokers compared to the broad reference group. Our observation is in line with what has been previously reported¹⁴⁰ and points towards a role of chronic inflammation as a mediating factor between smoking and low levels of fructosamine.

Based on our study results, we could not exclude a potential adverse biologic effect of low fructosamine in itself but likely, a major part of the observed inverse association between fructosamine and mortality in this

population could be explained by smoking and a higher amount of systemic inflammation. These components may act either as confounding or mediation factors. A likely similar epiphenomenon was observed in the ARIC cohort.¹⁰⁵

Study IV

With this large prospective study, we found expected associations for established risk factors of T2D. In addition, we described the long-term trajectories of those factors, reflecting glycemic and lipid related exposure as well as inflammatory processes. The trajectories of the population mean values were different in T2D cases compared to control subjects many years before a diagnosis of T2D. This time span of pre-diagnostic long-term trajectories of risk factors have not previously been reported.

Risk factors, which are associated with reduced insulin sensitivity, e.g. BMI, triglycerides and uric acid, were higher in T2D cases than in control subjects up to 25 years before the diagnosis. These observed elevations, point towards an early presence of insulin resistance in subjects who much later are diagnosed with T2D. Because of a long-term presence of insulin resistance, the fasting glucose in those subjects starts to increase simultaneously. Therefore, a slightly increased fasting glucose might serve as an indicator of reduced insulin sensitivity and may not have autonomous casual properties.

We estimated absolute risk of T2D after stratifying on sex, age, BMI, triglycerides and

fasting glucose. Overweight, and in particular obesity, strongly increased the risk. This suggests that any weight reduction may imply protection of developing T2D, which also is well documented in primary prevention trials.^{153,154} In addition, weight reduction with bariatric surgical procedures, was reported to lower the risk of T2D independently of baseline BMI.¹⁵⁵ The total incidence of T2D was higher for men than for women, but at corresponding levels of the risk factors, the risk was comparable in both genders. Hence, a greater propensity to dyslipidemia, overweight and obesity was apparent in men. This might relate to a tendency for women to have better protection, compared to men, in developing intraabdominal obesity, also when men and women adopt comparable physical inactivity and poor dietary habits.¹⁵⁶

The risk prediction model we developed, including only a few parameters, which are commonly available in clinical practice, performed well compared to several other risk scores,¹⁵⁷ in discriminating cases from non-cases.

We did not include fructosamine in the risk prediction model. However, we investigated the role of fructosamine in the development towards a diagnosis of T2D. We observed a crude 16% increased hazard for one standard deviation change. When we adjusted for triglycerides, only a marginal association remained. For cut-offs derived to reflect diagnostic levels for prediabetes and diabetes, fructosamine was positively associated with the incidence of T2D. This may suggest

fructosamine as a predictor of T2D.

Fructosamine showed no difference between cases and controls during the early years of the trajectory, though there was a difference at 15 years before a diagnosis. This point in time coincides with an average glucose indicating prediabetes. In fact, this would be in accordance with the results from study I,¹³⁷ where we reported no correlation of the glycemic markers in states of normal glucose tolerance.

As discussed in the opening background chapter of this book, the ARIC study found a five-fold increased risk for individuals above the 95th percentile of fructosamine, i.e. ≥ 2.64 mmol/L.¹¹⁵ In the AMORIS study, we found an increased risk for those above the 95th percentile of fructosamine (> 2.40 mmol/L) but the magnitude of this elevated risk was smaller. Methodological issues in the ascertainment of diagnoses could potentially explain parts of the difference. On average, the AMORIS cohort reported lower fructosamine levels compared to the ARIC study, which shows the heterogeneity of populations. Further, based on AMORIS results, individuals above 2.60 mmol/L are already in a hyperglycemic state¹³⁷ and accordingly should be excluded from studies of incident diabetes

Future perspectives for fructosamine

Among alternative tests other than glucose itself, HbA1c is the preferred test for the diagnosis as well as for long-term control of diabetes. Other tests of glycated proteins, including fructosamine, are rarely used in clinical practice. Growing evidence for the usefulness of fructosamine as a complementary biomarker for glucose metabolism is emerging.^{105,115,137,152,158} Despite the demonstrated advantages of the HbA1c test,²⁴ there are certain situations where HbA1c can fail to reflect glycemic exposure accurately,²⁴ e.g. in individuals with

any disturbance of erythrocyte turnover and hemoglobinopathies. In addition, unavailability of the test globally, may limit its use. In these situations, other measurements of long-term glycemic exposure merits attention and fructosamine might be an alternative after consideration of its disadvantages.^{94,96-99,159} Another possible limitation of the HbA1c test is its higher cost compared to an ordinary plasma/serum test. In large observational cohort studies as well as in pharmaceutical RCTs the cost/time reduction by exchanging HbA1c with fructosamine might be substantial.

CONCLUSIONS

Fructosamine is closely associated with HbA1c and glucose respectively and may be a useful biomarker of hyperglycemia and glucose control in clinical and epidemiological studies.

The associations were seen particularly in patients with type 1 or type 2 diabetes. Fructosamine and HbA1c tend to parallel each other over time in subjects with diabetes indicating similar reflections of glucose. The results also suggest that fructosamine discriminates well between subjects with and without diabetes.

High fructosamine levels are associated with an increased risk of acute myocardial infarction and death from any cause.

At high fructosamine levels, these strong associations remained after adjustment for major cardiovascular risk factors and glucose levels. Similar associations were obtained by using HbA1c as a predictor of risk. Fructosamine can be used either as a single prognostic tool or as a complement to HbA1c.

Low levels of fructosamine in individuals without diabetes were found to be associated with increased mortality.

Smoking and chronic inflammation seem to at least partially explain this association but an independent contribution by low fructosamine cannot be excluded.

Development of T2D is associated with subtle elevations of metabolic risk factors and inflammation more than 20 years before diagnosis.

This suggests that diabetogenic processes tied to chronic insulin resistance operate for decades prior to the development of T2D. A simple risk classification can help to early identify individuals at increased risk. Fructosamine was increased in cases of T2D compared to controls about 15 years before a diagnosis.

FUTURE STUDIES

In this thesis, studies were designed for evaluation of fructosamine in relation to established biomarkers of diabetes, to CHD and mortality and in relation to the diagnosis of T2D. Future research, based on the AMORIS cohort, intends to investigate the relationship of fructosamine with microvascular diseases, prevalent as well as incident, and in particular, diabetic retinopathy. Furthermore, better understanding of the relationship between fructosamine and HbA1c in anemic conditions, red blood cell disorders and in subgroups of subjects with disorders of the protein metabolism, warrants attention.

SVENSK SAMMANFATTNING

Diabetes är en sjukdom som definieras av högt blodsocker. Sjukdomen är ett stort hälsoproblem globalt och ökar dramatiskt risken för hjärt-kärlsjukdomar och förtida död. För att diagnostisera diabetes och även för att kontrollera status hos diagnostiserade patienter, används idag i stort sett endast två metoder; mätning av fasteglukos i blodet och/eller mätning av andelen glykerat hemoglobin (HbA1c). Det senare ger en uppskattning av hur den genomsnittliga glukosnivån har varit de senaste 2-3 månaderna medan fasteglukos ger en ögonblicksbild.

Denna avhandling har som målsättning att utvärdera fruktosamin, en annan indikator för genomsnittliga blodsockernivåer vilken dock avspeglar glukosnivåerna under de senaste 2-3 veckorna, och på så sätt bidra med mer kunskap om denna markör. Dessutom undersöks utvecklingen av flera riskfaktorer, varav fruktosamin är en, i ett 25-års perspektiv som riskprediktor av typ 2 diabetes.

I den första studien, jämförde vi de två etablerade metoderna för diagnos och kontroll av diabetes (fasteglukos och HbA1c) med fruktosamin. Vi såg ett starkt linjärt samband mellan alla dessa tre markörer vid förhöjda glukosnivåer. Vidare förändrades markörerna likartat över tid oavsett ökning eller sänkning av nivåerna av de tre olika riskmarkörerna. Fruktosamin visade sig också förtjänstfullt kunna skilja ut individer med högt blodsocker från individer med normala nivåer.

I den andra studien undersökte vi om det fanns samband mellan förhöjda fruktosaminvärden och risken för akut hjärtinfarkt och förtida död. I modeller där hänsyn togs till kön, ålder, socioekonomisk situation och blodfetter, såg vi en fördubblad risk för både akut hjärtinfarkt och död

vilken kunde hänföras till bristande glukoskontroll. Även när hänsyn togs till glukosnivåer hade fruktosamin en tilläggs effekt för bedömning av dessa händelser. Vi konstaterade vidare att fruktosamin och HbA1c var lika goda prediktorer av framtida hjärt-kärlsjukdom och död.

I den tredje studien var fokus på lägre fruktosaminnivåer hos individer som inte hade diagnostiserad diabetes. Vi såg ett samband mellan låga till moderata nivåer och en ökad dödlighet. Baserat på känslighetsanalyser tror vi att detta samband åtminstone till en del beror på skillnader i rökvanor och kronisk inflammation men en biologiskt relaterad skadlig effekt av det låga fruktosaminet kan inte uteslutas.

Vi såg i den fjärde studien att BMI, blodfetter och inflammationsmarkörer var genomsnittligt högre hos framtida diabetesfall jämfört med matchade referensindivider mer än 20 år före diagnos. Samtidigt var även fasteglukos högre hos fallen om än inte lika uttalat. Vi konstruerade en risktabell baserad på kön, ålder, BMI, blodfetter och fasteglukos som på ett översiktligt sätt kan underlätta vid en bedömning av en individs långsiktiga risk att utveckla typ 2 diabetes.

Sammanfattningsvis visar avhandlingens studier på att utvecklingen av typ 2 diabetes börjar tidigt och att ett fåtal faktorer på ett enkelt sätt kan identifiera individer under hög risk. Vi visade en generell användbarhet av fruktosamin som ett kliniskt och epidemiologiskt verktyg eftersom det avspeglar den underliggande blodsockerbelastningen på ett relevant sätt och komplementärt till etablerade biomarkörer. Sambandet med hjärtinfarkter och förtida död visar även på en betydelse av fruktosamin som kardiovaskulär riskfaktor.

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